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(54) Title: MUTANT FORMS OF CHOLERA HOLOTOXIN AS AN ADJUVANT

(57) Abstract: Mutant cholera holotoxins comprising a cholera toxin subunit (A) having single amino acid substitutions in the amino acid positions (16 or 72) or a double amino acid positions (16 and 68) or (68 and 72) have reduced toxicity compared to the wild-type cholera holotoxin. The mutant cholera holotoxins are useful as adjuvants in immunogenic compositions to enhance the immune response in a vertebrate host to a selected antigen from a pathogenic bacterium, virus, fungus, or parasite, a cancer cell, a tumor cell, an allergen, or a self-molecule.

MUTANT FORMS OF CHOLERA HOLOTOXIN AS AN ADJUVANT

CROSS-REFERENCE TO OTHER APPLICATIONS

5 This application claims the benefit of the priority of United States provisional patent application No. 60/296,537, filed June 7, 2001.

BACKGROUND OF THE INVENTION

The body's immune system activates a variety of mechanisms for attacking 10 pathogens (Janeway, Jr, CA. and Travers P., eds., in *Immunobiology*, "The Immune System in Health and Disease," Second Edition, Current Biology Ltd., London, Great Britain (1996)). However, not all of these mechanisms are necessarily activated after immunization. Protective immunity induced by immunization is dependent on the capacity of an immunogenic composition to elicit the appropriate immune response to 15 resist or eliminate the pathogen. Depending on the pathogen, this may require a cell-mediated and/or humoral immune response.

Many antigens are poorly immunogenic or non-immunogenic when administered by themselves. Strong adaptive immune responses to antigens almost 20 always require that the antigens be administered together with an adjuvant, a substance that enhances the immune response (Audbert, F.M. and Lise, L.D. 1993 *Immunology Today*, 14: 281-284).

The need for effective immunization procedures is particularly acute with respect to infectious organisms that cause acute infections at, or gain entrance to the body through, the gastrointestinal, pulmonary, nasopharyngeal or genitourinary 25 surfaces. These areas are bathed in mucus, which contains immunoglobulins consisting largely of secretory immunoglobulin IgA (Hanson, L.A., 1961 *Intl. Arch. Allergy Appl. Immunol.*, 18, 241-267; Tomasi, T.B., and Zigelbaum, S., 1963 *J. Clin. Invest.*, 42, 1552-1560; Tomasi, T.B., et al., 1965 *J. Exptl. Med.*, 121, 101-124). This immunoglobulin is derived from large numbers of IgA-producing plasma cells, which 30 infiltrate the lamina propria regions underlying the mucosal membranes (Brandtzaeg, P., and Baklein, K., 1976 *Scand. J. Gastroenterol.*, 11 (Suppl. 36), 1-45; and

Brandtzaeg, P., 1984 "Immune Functions of Human Nasal Mucosa and Tonsils in Health and Disease", page 28 *et seq.* in *Immunology of the Lung and Upper Respiratory Tract*, Bienenstock, J., ed., McGraw-Hill, New York, NY). The secretory immunoglobulin IgA is specifically transported to the luminal surface through the action of the secretory component (Solari, R., and Kraehenbuhl, J-P, 1985 *Immunol. Today*, 6, 17-20).

Parenteral immunization regimens are usually ineffective in inducing secretory IgA responses. Secretory immunity is most often achieved through the direct immunization of mucosally associated lymphoid tissues. Following their induction at one mucosal site, the precursors of IgA-producing plasma cells extravasate and disseminate to diverse mucosal tissues where final differentiation to high-rate IgA synthesis occurs (Crabbe, P.A., *et al.*, 1969 *J. Exptl. Med.*, 130, 723-744; Bazin, H., *et al.*, 1970 *J. Immunol.*, 105, 1049-1051; Craig, S.W., and Cebra, J.J., 1971 *J. Exptl. Med.*, 134, 188-200). Extensive studies have demonstrated the feasibility of mucosal immunization to induce this common mucosal immune system (Mestecky, J., *et al.*, 1978 *J. Clin. Invest.*, 61, 731-737), but with rare exceptions the large doses of antigen required to achieve effective immunization have made this approach impractical for purified antigens.

Among the strategies investigated to overcome this problem is the use of mucosal adjuvants. A number of adjuvants that enhance the immune response of antigens are known in the prior art (Elson, C. O., and Ealding, W., 1984 *J. Immunol.*, 132, 2736-2741). These adjuvants, when mixed with an antigen, render the antigen particulate, helping retain the antigen in the body for longer periods of time, thereby promoting increased macrophage uptake and enhancing immune response. However, untoward reactions elicited by many adjuvants or their ineffectiveness in inducing mucosal immunity have necessitated the development of better adjuvants for delivery of immunogenic compositions. Unfortunately, adjuvant development to date has been largely an empirical exercise (Janeway, Jr., *et al.*, cited above at pages 12-25 to 12-35). Thus, a rational and a more direct approach is needed to develop effective adjuvants for delivery of antigenic compositions.

It has been reported that the toxin secreted by the Gram-negative bacterium *Vibrio cholerae* (*V. cholerae*), the causative agent of the gastrointestinal disease cholera, is extremely potent as an adjuvant. Cholera toxin (CT) has been reported as a 382 amino acid sequence (SEQ ID NO: 1) (Mekalanos, J.J., *et al.*, 1983 *Nature*, 306, 551-557), which has an 18 amino acid signal (amino acids 1 to 18 of SEQ ID NO: 1).
5 The cholera toxin holotoxin molecule is a hexaheteromeric complex that consists of a peptide subunit designated CT-A (SEQ ID NO: 2 or amino acids 19 to 258 of SEQ ID NO: 1), which is responsible for the enzymatic activity of the toxin, and five identical peptide subunits, each designated CT-B (each having a 21 amino acid signal (amino acids 259 to 379 of SEQ ID NO: 1), followed by the CT-B peptide subunit (amino acids 280-382 of SEQ ID NO: 1)), which is involved in the binding of the toxin to the intestinal epithelial cells as well as other cells which contain ganglioside GM₁ on their surface (Gill, D.M., 1976 *Biochem.*, 15, 1242-1248; Cuatrecasas, P., 1973 *Biochem.*, 12, 3558-3566). CT produced by *V. cholerae* has the CT-A subunit proteolytically
10 cleaved within the single disulfide-linked loop between the cysteines at amino acid positions 187 and 199 of the mature CT-A (SEQ ID NO: 2) to produce an enzymatically active A1 polypeptide (Kassis, S., *et al.*, 1982 *J. Biol. Chem.*, 257, 12148-12152), and a smaller polypeptide A2, which links fragment A1 to the CT-B pentamer (Mekalanos, J.J., *et al.*, 1979 *J. Biol. Chem.*, 254, 5855-5861). Toxicity
15 results when the enzymatically active fragment CT-A1, upon entry into enterocytes, ADP-ribosylates a regulatory G-protein (G_sα). This leads to constitutive activation of adenylate cyclase, increased intracellular concentration of cAMP, and secretion of fluid and electrolytes into the lumen of the small intestine, thereby causing toxicity (Gill, D.M., and Meren, R., 1978 *Proc. Natl. Acad. Sci., USA*, 75, 3050-3054). *In vitro*,
20 ADP-ribosyl transferase activity of CT is stimulated by the presence of accessory proteins called ARFs, small GTP-binding proteins known to be involved in vesicle trafficking within the eukaryotic cell (Welsh, C.F., *et al.*, "ADP-Ribosylation Factors: A Family of Guanine Nucleotide-Binding Proteins that Activate Cholera Toxin and Regulate Vesicular Transport", pages 257-280 in *Handbook of Natural Toxins: Bacterial Toxins and Virulence Factors in Disease Vol. 8* (Moss, J., *et al.*, eds., Marcel Dekker, Inc., New York, NY (1995)).
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Co-administration of CT with an unrelated antigen has been reported to result in the induction of concurrent circulating and mucosal antibody responses to that antigen (Mekalanos, J.J., *et al.*, 1983 *Nature*, 306, 551-557). To minimize the occurrence of undesirable symptoms such as diarrhea caused by wild-type CT in humans, it would be preferable to use as an adjuvant a form of the CT holotoxin that has substantially reduced toxicity. Mutants of CT have been suggested as a means for achieving a more useful adjuvant. One way to rationally design mutant cholera toxin holotoxins (CT-CRMs) with substantially reduced toxicity is to identify and alter amino acid residues in the toxin molecule that are completely conserved in the family of cholera (CT) and related heat-labile enterotoxins (LT-I, LT-IIa and LT-IIb) of *E. coli*. Another rational way to generate mutant CT-CRMs with substantially reduced toxicity is to alter amino acid residues in the holotoxin molecule that have been identified as being important for NAD-binding based on the structural alignment of the CT backbone with the backbone of related toxins possessing ADP-ribosyl transferase enzyme activity such as diphtheria toxin (DT) and pertussis toxin (PT) (Holmes, R. K., "Heat-labile enterotoxins (*Escherichia coli*)" in *Guidebook to Protein Toxins and their Use in Cell Biology*, Montecucco, C. and Rappnoli, R., Eds., Oxford Univ. Press, Oxford, England (1997); and Holmes, R. K. *et al.*, "Cholera toxins and related enterotoxins of Gram-negative bacteria", pp. 225-256 in *Handbook of Natural Toxins: Bacterial Toxins and Virulence Factors in Disease*, vol. 8, Moss, J., *et al*, Eds., Marcel Dekker, Inc., New York, NY 1995).

Recently, one such rationally-designed, genetically-detoxified mutant of CT was disclosed wherein a single nonconservative amino acid substitution (glutamic acid to histidine) was introduced by altering the amino acid at position 29 in the A subunit (designated CT-CRM_{E29H}). The resulting mutant cholera holotoxin demonstrated substantially reduced enzymatic toxicity, but with superior adjuvanting and immunogenic properties (International Patent Publication No. WO 00/18434, incorporated in its entirety by reference).

Thus, there is a need to identify and/or rationally design additional mutant forms of the CT holotoxin that have substantially reduced toxicity, yet possess the same or enhanced adjuvanting properties as the wild-type CT holotoxin.

SUMMARY OF THE INVENTION

In one aspect, this invention provides novel mutant, immunogenic forms of cholera holotoxin (CT-CRMs) having significantly reduced toxicity compared to a wild-type CT, but which retain their ability as powerful stimulators of the immune system. Specifically, the invention pertains to four mutant cholera holotoxins (CT-CRMs), desirably generated by site-directed mutagenesis and having substantially reduced toxicity compared to a wild-type CT, but with no loss in adjuvanting properties.

In one embodiment, a novel CT-CRM of this invention comprises the amino acid sequence of CT subunit A or a fragment thereof, wherein the amino acid residue in the amino acid position 16 of the A subunit is substituted with another amino acid which substitution results in a substantial reduction in toxicity. In a preferred embodiment of the invention, the amino acid isoleucine at amino acid position 16 of the A subunit is substituted with an alanine. For determination of the amino acid position, the sequence of CT-A is exemplified in SEQ ID NO: 2. However, other variants and fragments of CT-A may also be employed.

In another embodiment, a novel CT-CRM of this invention comprises the amino acid sequence of CT subunit A or a fragment thereof, wherein the amino acid residue in the amino acid position 72 of the A subunit is substituted with another amino acid which substitution results in a substantial reduction in toxicity. In a preferred embodiment of the invention, the amino acid valine at the amino acid position 72 of the A subunit is substituted with a tyrosine.

In another embodiment, a novel immunogenic, mutant CT-CRM of this invention has substantially reduced CT toxicity and comprises the amino acid sequence of subunit A of CT or a fragment thereof, wherein both amino acid residues in the amino acid positions 16 and 68 in the A subunit are substituted with amino acids different from that present in amino acid positions 16 and 68 of wild-type CT, which substitutions result in a substantial reduction in toxicity. In a preferred embodiment of this aspect of the invention, the amino acid alanine is substituted for isoleucine at the amino acid position 16 in the A subunit, and the amino acid tyrosine is substituted for serine at amino acid position 68 in the A subunit.

In yet another embodiment, a novel immunogenic, mutant CT-CRM of this invention has substantially reduced CT toxicity and comprises the amino acid sequence of subunit A of CT or a fragment thereof, wherein both amino acid residues in the amino acid positions 68 and 72 in the A subunit are substituted with amino acids different from that present in amino acid positions 68 and 72 of wild-type CT, which substitutions result in a substantial reduction in toxicity. In a preferred embodiment of this aspect of the invention, the amino acid tyrosine is substituted for serine at amino acid position 68 of the A subunit, and the amino acid tyrosine is substituted for valine at amino acid position 72 of the A subunit.

10 In another aspect, the invention provides a method for producing the novel CT-CRMs described above by employing site-directed mutagenesis of the DNA encoding the A subunit in the wild-type CT using conventional techniques, such that the mutagenized CT now has substantially reduced toxicity without compromising the toxin's ability to stimulate an immune response.

15 In yet another aspect of the invention, there is provided an immunogenic composition comprising a selected antigen, a mutant CT-CRM as described above as an adjuvant to enhance the immune response in a vertebrate host to the antigen, and a pharmaceutically acceptable diluent, excipient or carrier. Preferably, the CT-CRM is useful for the generation or enhancement of systemic and/or mucosal antigenic immune responses in a vertebrate host to the selected antigen. The selected antigen may be a polypeptide, peptide or fragment derived from a pathogenic virus, bacterium, fungus or parasite. The selected antigen may be a polypeptide, peptide or fragment derived from a cancer cell or tumor cell. The selected antigen may be a polypeptide, peptide or fragment derived from an allergen so as to interfere with the production of IgE so as to moderate allergic responses to the allergen. The selected antigen may be a polypeptide, peptide or fragment derived from a molecular portion thereof which represents those produced by a host (a self molecule) in an undesired manner, amount or location, such as those from amyloid precursor protein, so as to prevent or treat disease characterized by amyloid deposition in a vertebrate host.

20 25 30 In still another aspect, this invention provides a method for using these CT-CRMs as adjuvants in immunogenic compositions or methods for increasing the ability

of an antigenic composition containing a selected antigen as described above to elicit an immune response in vertebrate host by including an effective adjuvanting amount of one or more of the novel detoxified mutant cholera holotoxins (CT-CRMs) described above.

5 In yet a further aspect of the invention, there are provided DNA sequences encoding the novel immunogenic, mutant CT-CRMs with substantially reduced toxicity as described above. Preferably, the DNA sequence(s) encodes for both the mutant A subunit with reduced toxicity and subunit B. Alternatively, the DNA sequence may encode only the mutant A subunit with reduced toxicity, where the mutant CT-A is
10 fused with an additional binding domain, or is co-expressed with LT-B and allowed to co-assemble.

In a further aspect of the invention, there is provided a plasmid containing isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic, detoxified, mutant cholera holotoxin as described herein, and wherein
15 such a DNA sequence is operatively linked to regulatory sequences which direct expression of the CT-CRM in a host cell. Preferably the regulatory sequences comprise an arabinose inducible promoter. In one embodiment of this aspect, the invention relates to a plasmid, designated pLP903, that contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic mutant
20 CT-CRM with substantially reduced toxicity wherein the amino acid alanine is substituted for isoleucine at amino acid position 16 in the A subunit. In a second embodiment of this aspect, the invention relates to a plasmid, designated pLP905, that contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic mutant CT-CRM with substantially reduced toxicity wherein the
25 amino acid tyrosine is substituted for valine at the amino acid position 72 in the A subunit. In a third embodiment of this aspect, the invention relates to a plasmid, designated pLP904, that contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic, mutant CT-CRM with substantially reduced toxicity wherein the amino acid alanine is substituted for isoleucine at amino acid position 16, and amino acid tyrosine is substituted for serine at amino acid position 68 in the A subunit. In yet an additional embodiment of this aspect, the invention relates
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to a plasmid, designated pLP906, that contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic, mutant CT-CRM with substantially reduced toxicity wherein the amino acid tyrosine is substituted for serine at the amino acid position 68, and amino acid tyrosine is substituted for valine at amino acid position 72 in the A subunit.

In a further aspect of the invention, there is provided a suitable host cell line transformed, infected, transduced or transfected with a plasmid as described herein. The immunogenic, detoxified, mutant cholera holotoxins are produced by transforming, infecting, transducing or transfecting a suitable host cell with one of the 10 plasmids described above and culturing the host cell under culture conditions which permit the expression by the host cell of said recombinant immunogenic, mutant cholera holotoxin protein with substantially reduced toxicity.

These and other aspects of the invention will be apparent to one of skill in the art upon reading of the following detailed description of the invention.

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DETAILED DESCRIPTION OF THE INVENTION

Mutant forms of cholera holotoxin that exhibit reduced toxicity, but which retain their superior adjuvanting properties, and the utility of these mutant forms of CTs as adjuvants in immunogenic compositions are described herein.

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A. *Mutant, Detoxified Cholera Toxin Holotoxins*

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Novel mutant, detoxified immunogenic forms of cholera holotoxin (CT-CRMs) of this invention are characterized by significantly reduced toxicity compared to a wild-type CT. However, such CT-CRMs retain their ability as powerful stimulators of the immune system. The CT-CRMs of this invention are characterized by one or several amino acid substitutions in the mature CT-A subunit of cholera toxin. The various mutant CT-A subunits of this invention also retained their ability to assemble with CT-B subunits to form mutant CT holotoxins that resembled wild-type CT in adjuvanticity, but which exhibited substantially reduced toxicity compared to the wild-type CT. The CT-CRMs of this invention may employ mutant or altered CT-A subunits associated with wild-type CT-B subunits to create a functional holotoxin. Alternatively, the CT-

CRMs of this invention may comprise the altered or mutated CT-A subunits associated with altered or mutated CT-B subunits.

For determination of the amino acid position numbers describing the locations of the amino acid substitutions in the CT-CRMs of this invention, the sequence of mature CT-A is exemplified as SEQ ID NO: 2, i.e., amino acids 19-258 of SEQ ID NO: 1, a wild-type CT sequence. The nucleotide sequence encoding the A subunit of the cholera holotoxin is set forth in International patent publication No. WO 93/13202. Similarly, a suitable mature CT-B sequence may be illustrated by amino acids 280-382 of SEQ ID NO: 1. However, other variants, biotypes and fragments of CT-A and CT-B of *V. cholerae* may also be employed as sequences containing the amino acid substitutions described herein. See, for example, the ELTOR biotype of C. Shi *et al*, 1993 *Sheng Wu Hua Hsueh Tsa Chih*, 9(4):395-399; NCBI database locus No. AAC34728, and other sources of variants of *V. cholerae* toxin.

Preferably, the amino acid substitutions resulting in the CT-CRMs of this invention are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, i.e. conservative amino acid replacements. "Conservative" amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, tryptophan, and methionine; polar/neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. This invention is exemplified by CT-CRMs having a single amino acid substitution at either amino acid position 16 or at amino acid position 72 or double amino acid substitution at amino acid positions 16 and 68 or 68 and 72, as summarized in Table 1.

Table 1: Single and Double CT-CRM Mutants

Amino Acid Substitution	Native	Mutant	Abbreviation
16	Isoleucine ₁₆	Alanine ₁₆	CT-CRM _{I16A}
72	Valine ₇₂	Tyrosine ₇₂	CT-CRM _{V72Y}
16 and 68	Isoleucine ₁₆	Alanine ₁₆	CT-CRM _{I16A,S68Y}
	Serine ₆₈	Tyrosine ₆₈	
68 and 72	Serine ₆₈	Tyrosine ₆₈	CT-CRM _{S68Y,V72Y}
	Valine ₇₂	Tyrosine ₇₂	

Thus, in one embodiment, a novel CT-CRM of this invention comprises the amino acid sequence of CT subunit A or a fragment thereof, wherein the amino acid residue in the amino acid position 16 of the A subunit is substituted with another amino acid which substitution results in a substantial reduction in toxicity. In a preferred embodiment of the invention, the amino acid isoleucine at amino acid position 16 of the A subunit is substituted with an alanine. This CT-CRM_{I16A} demonstrates superior adjuvanting properties.

In another embodiment, a novel CT-CRM of this invention comprises the amino acid sequence of CT subunit A or a fragment thereof, wherein the amino acid residue in the amino acid position 72 of the A subunit is substituted with another amino acid, which substitution results in a substantial reduction in toxicity. In a preferred embodiment of the invention, the amino acid valine at the amino acid position 72 of the A subunit is substituted with a tyrosine, resulting in CT-CRM_{V72Y}. This CT-CRM_{V72Y} demonstrates superior adjuvanting properties.

In another embodiment, a novel immunogenic, mutant CT-CRM of this invention has substantially reduced CT toxicity and comprises the amino acid sequence of subunit A of CT or a fragment thereof, wherein both amino acid residues in the amino acid positions 16 and 68 in the A subunit are substituted with amino acids different from that present in amino acid positions 16 and 68 of wild-type CT, which substitutions result in a substantial reduction in toxicity. In a preferred embodiment of this aspect of the invention, the amino acid alanine is substituted for isoleucine at the amino acid position 16 in the A subunit, and the amino acid tyrosine is substituted for

serine at amino acid position 68 in the A subunit, resulting in CT-CRM_{I16A, S68Y}, which demonstrates superior adjuvanting properties

In yet another embodiment, a novel immunogenic, mutant CT-CRM of this invention has substantially reduced CT toxicity and comprises the amino acid sequence of subunit A of CT or a fragment thereof, wherein both amino acid residues in the amino acid positions 68 and 72 in the A subunit are substituted with amino acids different from that present in amino acid positions 68 and 72 of wild-type CT, which substitutions result in a substantial reduction in toxicity. In a preferred embodiment of this aspect of the invention, the amino acid tyrosine is substituted for serine at amino acid position 68 of the A subunit, and the amino acid tyrosine is substituted for valine at amino acid position 72 of the A subunit. This CT-CRM_{S68Y, V72Y} demonstrates superior adjuvanting properties.

The phenotypic effects of the novel CT-CRMs of Table 1 on the structure and function of CT were assessed. The mutant A subunits generated by site directed mutagenesis of the CT-encoding gene were also able to assemble into immunoreactive holotoxin in the presence of subunit B as determined by non-denaturing gel electrophoresis assay (*see* Table 3, Example 2). Each mutant holotoxin was also tested in a Y-1 adrenal tumor cell assay to determine its residual toxicity compared to wild-type CT holotoxin (*see* Tables 4 and 5, Example 3). The results presented in Table 4 demonstrate that the mutant CT-CRMs had substantially reduced toxicity when compared with wild-type cholera holotoxin. The residual toxicities of the CT-CRMs with single and double amino acid substitutions were substantially reduced in comparison to that of the wild-type CT.

Each of the mutant CT-CRMs was also compared to wild-type CT in an ADP-ribosyltransferase activity assay (*See* Example 4). The results, which were generally in agreement with the toxicity data generated in the Y-1 adrenal cell assay, indicated that the ADP-ribosyltransferase activity of the various CT-CRMs was substantially diminished when compared to wild-type CT (Table 6). The mutant with the largest ADP-ribosyl- transferase activity appeared to be the double mutant CT-CRM_{I16A, S68Y}. This activity was approximately only 3.3% of wild-type CT. The enzyme activity of

CT-CRMs, CT-CRM_{V72Y}, CT-CRM_{I16A}, and CT-CRM_{S68Y,V72Y}, were 1.1%, 2.4% and 1.2% respectively of the activity of the wild-type CT.

Still other CT-CRMs of this invention may contain at least the single or double mutations described specifically above and at least one additional mutation at a position other than at one or more of the amino acid residues 16, 68, or 72 as set forth above. International patent publication No. WO 93/13202, which is hereby incorporated by reference, describes a series of mutations in the CT-A subunit that serve to reduce the toxicity of the cholera holotoxin. These mutations include making substitutions for the arginine at amino acid 7, the aspartic acid at position 9, the arginine at position 11, the glutamic acid at position 29, the histidine at position 44, the valine at position 53, the arginine at position 54, the serine at position 61, the serine at position 63, the histidine at position 70, the valine at position 97, the tyrosine at position 104, the proline at position 106, the histidine at position 107, the glutamic acid at position 110, the glutamic acid at position 112, the serine at position 114, the tryptophan at position 127, the arginine at position 146 and the arginine at position 192. International patent publication No. WO 98/42375, which is hereby incorporated by reference, describes making a substitution for the serine at amino acid 109 in the A subunit, which serves to reduce the toxicity of the cholera holotoxin.

Other useful CT-CRM mutant proteins useful in this invention include a full-length holotoxin with one or more of the specific mutations provided above, a polypeptide or a fragment thereof containing the mutagenized residues described above and which protein, polypeptide or fragment retains the adjuvanticity of wild-type CT from which it is derived, but is characterized by reduced toxicity.

Immunologically active fragments of these CT-CRMs with reduced enzymatic activity may also be useful in the methods and compositions of this invention. Fragments ordinarily will contain at least at least about 25 contiguous amino acids of the CT-CRM proteins containing the site of mutagenesis noted above. More typically a CT-CRM fragment contains at least about 75 contiguous amino acids. Another fragment of a CT-CRM contains at least about 100 contiguous amino acids. Still another embodiment of a CT-CRM subunit A contains at least about 150 contiguous amino acids in length.

A fragment of the CT-CRMs described herein is useful in the methods and compositions described below if it generates or enhances the immune response to selected antigens in the vertebrate host. Fragments include truncations of the carboxy-terminal region of the CT-CRMs. For example, a CT-CRM truncated so that it
5 contains only a CT-A mutant subunit is a desirable fragment. Similarly, CT-A subunits truncated at about residues 240 or 250 are desirable fragments. Still other fragments CT-CRMs of this invention may be selected. Additional fragments of the CT-CRM holotoxin may contain less than five repetitions of the CT-B subunits or truncated CT-B subunits. The foregoing fragments may also contain one or more of the specific
10 mutations described above.

Other suitable CT-CRM proteins may include those in which one or more of the amino acid residues includes a substituted group. Still another suitable CT-CRM holotoxin protein is one in which the CT-CRM polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for
15 example, polyethylene glycol). Another suitable CT-CRM protein is one in which additional amino acids are fused to the polypeptide, such as a leader or secretory sequence, or a sequence which is employed to enhance the immunogenicity of the CT-CRM protein. Still other modifications of the CT-CRMs include the above-mentioned deletion of the CT-A signal or leader sequence at the N terminus of CT, i.e., amino
20 acids 1-18 of SEQ ID NO: 1 and/or the deletion of the CT-B signal or leader sequence at amino acids 259-279 of SEQ ID NO: 1, and/or the deletion of other regions that do not effect immunogenicity. Similarly, a modification of the CT-CRMs described herein includes include replacing either signal or leader sequence with another signal or leader sequence. See, e.g., US Patent No. 5,780,601, incorporated by reference herein.

25 Still another example of suitable CT-CRM proteins are those in which optional amino acids (e.g., -Gly-Ser-) or other amino acid or chemical compound spacers may be included at the termini of the polypeptide for the purpose of linking multiple holotoxin proteins together or to a carrier. For example, useful CT-CRMs may include one or more of the above-described CT-CRMs coupled to a carrier protein.
30 Alternatively, a useful CT-CRM may be present in a fusion protein containing multiple CT-CRMs, optionally coupled to carrier protein.

For these embodiments, the carrier protein is desirably a protein or other molecule that can enhance the immunogenicity of the selected CT-CRM. Such a carrier may be a larger molecule that also has an adjuvanting effect. Exemplary conventional protein carriers include, without limitation, *E. coli* DnaK protein, galactokinase (GalK, which catalyzes the first step of galactose metabolism in bacteria), ubiquitin, α -mating factor, β -galactosidase, and influenza NS-1 protein.

5 Toxoids (i.e., the sequence which encodes the naturally occurring toxin, with sufficient modifications to eliminate its toxic activity) such as diphtheria toxoid and tetanus toxoid, their respective toxins, and any mutant forms of these proteins, such as CRM₁₉₇ (a non-toxic form of diphtheria toxin, see US Patent No. 5,614,382), may also be employed as carriers. Other carriers include exotoxin A of *Pseudomonas aeruginosa*, heat labile toxins of *E. coli* and rotaviral particles (including rotavirus and VP6 particles). Alternatively, a fragment or epitope of the carrier protein or other immunogenic protein may be used. For example, a hapten may be coupled to a T cell

10 epitope of a bacterial toxin. See US Patent No 5,785,973. Similarly a variety of bacterial heat shock proteins, e.g., mycobacterial hsp-70 may be used. Glutathione-S-transferase (GST) is another useful carrier. One of skill in the art can readily select an appropriate carrier for use in this context. The fusion proteins may be formed by standard techniques for coupling proteinaceous materials. Fusions may be expressed

15 from fused gene constructs prepared by recombinant DNA techniques as described below.

20

Other suitable CT-CRMs described herein can differ from the specifically exemplified CT-CRMs by modifications that do not revive enzymatic toxicity, and do not diminish adjuvanticity, or by combinations of such attributes. For example, conservative amino acid changes may be made, which, although they alter the primary sequence of the CT-CRM protein, do not normally alter its function. In making such changes, the hydropathic index of amino acids can be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a polypeptide is generally understood in the art (Kyte & Doolittle, 1982 *J. Mol. Biol.*, 25 157(1):105-132). It is known that certain amino acids can be substituted for other amino acids having a similar hydropathic index or score and still result in a polypeptide

with similar biological activity. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. Those indices are:
5 isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is believed that the relative hydropathic character of the amino acid residue determines the secondary and tertiary structure of the resultant polypeptide, which in turn defines the interaction of the polypeptide with other molecules, such as enzymes, 10 substrates, receptors, antibodies, antigens, and the like. It is known in the art that an amino acid can be substituted by another amino acid having a similar hydropathic index and still obtain a functionally equivalent polypeptide. In such changes, the substitution of amino acids whose hydropathic indices are within +/-2 is preferred, those within +/-1 are particularly preferred, and those within +/-0.5 are even more particularly 15 preferred.

Substitution of like amino acids can also be made on the basis of hydrophilicity, particularly where the biologically functional equivalent polypeptide or peptide thereby created is intended for use in immunological embodiments. U.S. Patent No. 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of 20 a polypeptide, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *i.e.* with a biological property of the polypeptide.

As detailed in U.S. Patent No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate 25 (+3.0 ±1); glutamate (+3.0 ±1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); proline (-0.5 ±1); threonine (-0.4); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a 30 biologically equivalent, and in particular, an immunologically equivalent polypeptide. In such changes, the substitution of amino acids whose hydrophilicity values are within

± 2 is preferred; those within ± 1 are particularly preferred; and those within ± 0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

In addition, modifications, which do not normally alter the primary sequence of the CT-CRM protein, include *in vivo* or *in vitro* chemical derivatization of polypeptides, *e.g.*, acetylation, methylation, or carboxylation. Also included as CT-CRMs of this invention are these proteins modified by glycosylation, *e.g.*, those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; or by exposing the polypeptide to enzymes which affect glycosylation, such as mammalian glycosylating or deglycosylating enzymes. Also embraced as CT-CRMs are the above-identified mutagenized sequences, which have phosphorylated amino acid residues, *e.g.*, phosphotyrosine, phosphoserine, or phosphothreonine.

Also included as CT-CRMs of this invention are the above sequences that have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties. Among such CT-CRMs are included those containing residues other than naturally occurring L-amino acids, *e.g.*, D-amino acids or non-naturally occurring synthetic amino acids. Among other known modifications which may be present in CT-CRMs of the present invention are, without limitation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation,

proteolytic processing, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

The mutant CT-CRMs of this invention are thus holotoxins and exhibit reduced toxicity or are substantially less toxic than wild-type CT. As used herein, the terms and phrases "the holotoxin has reduced toxicity" or "substantially less toxic" or the like mean that the CT-CRM mutant of this invention, such as the four CT-CRM mutants described herein (CT-CRM_{H16A}, CT-CRM_{V72Y}, CT-CRM_{H16A, S68Y}, and CT-CRM_{S68Y, V72Y}), exhibits a substantially lower toxicity per unit of purified toxin protein compared to the wild-type CT. This "reduced toxicity" enables each mutant to be used as an adjuvant in an immunogenic composition without causing significant side effects, particularly those known to be associated with CT, e.g., diarrhea. As described in more detail below, the mutant CT-CRMs of this invention display significantly lower levels of toxicity than the wild-type CT in the Y-1 mouse adrenal cell assay, and a significantly reduced ADP-ribosyltransferase activity when compared to wild-type CT.

The immunogenic mutant CT-CRMs according to the present invention exhibit a balance of reduced toxicity and retained adjuvanticity, such that the resulting mutant CT protein functions as an adjuvant while being tolerated safely by the vertebrate host to which it is introduced. As indicated in the examples below, results in murine model assay systems indicate that the mutant CT-CRMs disclosed herein were able to significantly augment mucosal and systemic immune responses following intranasal administration of disparate antigens. Furthermore, even in the presence of pre-existing anti-CT immune responses, the mutant CT-CRMs were able to serve as efficient mucosal adjuvants. The studies which support these characteristics of the CT-CRMs of this invention are summarized below and more specifically stated in the Examples.

To evaluate the efficacy of the mutant CT-CRMs as mucosal adjuvants for compositions containing bacterial or viral antigens that have been identified as candidates for inclusion in immunogenic compositions, three disparate model antigen systems were examined: (1) the recombinant P4 outer membrane protein (also known as protein "e"(rP4)) of the nontypable *Haemophilus influenzae* bacterium (NTHi), (see U. S. Patent No. 5,601,831), (2) the native UspA2 outer membrane protein of the

5 *Moraxella catarrhalis* bacterium (International Patent Publication No. WO 98/28333), and (3) the native fusion glycoprotein (F protein) of respiratory syncytial virus (RSV) (see U.S. Patent No. 5,223,254). The mutant CT-CRMs were compared with each other, and to CT-CRM_{E29H} and the wild-type CT as an adjuvant for the NTHi rP4. In
10 a first study, the adjuvanting ability of the mutant CT-CRM_{II6A} to enhance the induction of systemic and mucosal antibodies to rP4 were assessed and compared with that of wild-type CT and CT-CRM_{E29H}. The results indicated that the CT-CRM_{II6A}, like the wild-type CT and CT-CRM_{E29H}, augmented the capacity of rP4 protein to elicit
15 systemic and humoral immune responses (see Tables 8 and 9). For example, six weeks after tertiary IN immunization, the anti-rP4 IgG antibody titers of mice immunized with rP4 protein formulated with either CT-CRM_{II6A} or CT-CRM_{E29H} were 40 times greater than that of mice immunized with the recombinant proteins in PBS alone. The antibody titers (IgG) of mice administered the recombinant protein plus wild-type CT holotoxin at a concentration of 1 µg were elevated 67-fold in comparison to antibody titers in
20 mice administered recombinant rP4 alone in saline six weeks after the primary IN immunization. The antibody titers of mice immunized with 1 µg of the mutant, CT-CRM_{E29H} were elevated 48-fold over antibody titers in mice immunized with rP4 alone. In comparison, the antibody titers of mice immunized with 1 µg and 0.1µg of the mutant, CT-CRM_{II6A}, were increased 15-fold and 27-fold respectively over the anti-rP4 antibody titers in mice immunized with rP4 alone in saline.

25 An examination of the protein-specific antibodies in the mucosal secretions two weeks after tertiary immunization further indicated that the CT-CRM_{II6A} facilitated the generation of local immune responses against the rP4 protein. Moreover, the anti-rP4 antibody titers were comparable to those induced by wild-type CT adjuvanted immunogenic composition (Table 9).

30 To test and compare the adjuvanting effects of mutant CT-CRMs in formulations containing 1 µg recombinant rP4 and 1 µg of one of the mutant CT-CRMs (CT-CRM_{II6A}, CT-CRM_{II6A,S68Y}, CT-CRM_{V72Y}, and CT-CRM_{S68Y,V72Y}) with a formulation containing 1 µg rP4 and 1 µg CT-CRM_{E29H}, or 1 µg rP4 alone in saline. The various compositions were delivered intranasally to female BALB/c mice, and the anti-rP4 IgG and IgA titers measured at weeks 3 and 5, and at week 5, day 6. The data

suggest that CT-CRMs, CT-CRM_{II6A} and CT-CRM_{V72Y} are as potent as CT-CRM_{E29H} in inducing systemic as well as mucosal anti-rP4 antibody response (Tables 10 and 11). The serum IgG titers of anti-rP4 antibody induced by the formulation containing rP4 and CT-CRM_{II6A} at week 5, day 6 was 22-fold greater than that induced by rP4 alone and half of the IgG levels induced by CT-CRM_{E29H}. However, serum IgG titers of anti-rP4 antibody induced by the formulation containing rP4 and CT-CRM_{V72Y} was 5 1.2-fold more than that induced by CT-CRM_{E29H} and 53-fold greater than that induced by rP4 alone. Although the rP4-specific IgG titers induced by CT-CRM_{II6A,S68Y} and CT-CRM_{S68Y,V72Y} were only approximately one-fifth of that induced by CT-CRM_{II6A} and CT-CRM_{V72Y}, these levels were still significantly higher than that induced by rP4 10 alone in saline.

The protein-specific IgA antibody titers in the sera of mice immunized with CT-CRMs, CT-CRM_{II6A}, CT-CRM_{II6A,S68Y}, CT-CRM_{V72Y} and CT-CRM_{S68Y,V72Y} were 6 to 23-fold greater than those of mice immunized IN with the rP4 alone.

15 The protein-specific IgA antibody titers in the bronchoalveolar wash, nasal wash, saliva and vaginal wash of mice immunized with CT-CRMs, CT-CRM_{II6A}, CT-CRM_{II6A,S68Y}, CT-CRM_{V72Y} and CT-CRM_{S68Y,V72Y} were comparable to the IgA levels in the mucosal wash pools of mice immunized with CT-CRM_{E29H}, but significantly greater than those of mice immunized with rP4 alone. (See Table 11).

20 In the above study, anti-rP4 antibody titers in the serum of each individual mouse in the six groups were also assessed. Specifically, 41 days following IN administration, IgA and IgG including IgG subclass IgG1, IgG2a, IgG2b and IgG3 endpoint titers were determined by ELISA. The results indicate that IgA and IgG subclass titers in each individual mouse receiving the formulation containing rP4 and 25 any one of the four mutant CT-CRMs were significantly higher than the IgA and IgG titers in animals receiving only the rP4 antigen in saline. (See Tables 12-17). The results further indicate that the IgA and IgG titers in animals receiving rP4 and one of the mutant CT-CRMs, CT-CRM_{II6A}, CT-CRM_{II6A,S68Y}, and CT-CRM_{V72Y}, were comparable to the IgA and IgG titers detected in mice receiving rP4 plus CT-CRM_{E29H}.

30 The capacity of the CT-CRMs of the present invention to augment systemic and mucosal immune responses against respiratory syncytial virus (RSV) glycoproteins

was examined using the purified native fusion (F) protein. Previously, it was demonstrated that BALB/c mice immunized IN with F protein adjuvanted with either CT or CT-CRM_{E29H} generated systemic and local IgG and IgA titers (Tebbey *et al*, cited above). This study also indicated that pre-existing anti-CT antibodies did not have a negative impact on the level of local or systemic anti-F protein IgA and IgG antibodies. Indeed, the study indicated that pre-existing anti-CT antibodies were beneficial for the generation of an augmented anti-F protein antibody response.

Additionally, the data also suggested a mechanism involving the neutralization of infectious virus by either mucosal or humoral immunoglobulins that are stimulated in response to the IN immunization protocol containing F/CT-CRM_{E29H}. In the present study, purified F protein (3 µg/dose) alone in saline or in a formulation containing 0.1 or 1 µg of the wild-type CT or 0.1 or 1 µg of one of the mutant CT-CRMs (CT-CRM_{E29H}, CT-CRM_{I16A}, CT-CRM_{I16A,S68Y}, CT-CRM_{V72Y} and CT-CRM_{S68Y,V72Y}) was administered IN to BALB/c mice. The protein-specific IgG and IgA antibody titers in the bronchoalveolar wash, nasal wash and vaginal wash of mice were determined. The protein-specific IgG and IgA antibody titers in the bronchoalveolar wash, nasal wash and vaginal wash of mice immunized with 1 µg of CT-CRM_{I16A}, CT-CRM_{I16A,S68Y}, CT-CRM_{V72Y} or CT-CRM_{S68Y,V72Y} were comparable to the IgG and IgA levels in the mucosal wash pools of mice immunized with wild-type CT or CT-CRM_{E29H}. (See Table 19). The mucosal protein-specific IgG levels in mice immunized with 0.1 µg of CT-CRM_{I16A}, CT-CRM_{I16A,S68Y}, CT-CRM_{V72Y} or CT-CRM_{S68Y,V72Y} though significantly higher than the levels detected in mice immunized with F-protein in saline, were nevertheless one-third to one-tenth less than the levels detected in mice immunized with 1 µg of the CT-CRMs. In contrast, significantly elevated levels of IgA in mucosal washes were only observed in mice immunized with 1 µg of the mutant CT-CRMs.

The capacity of mutant CT-CRMs to augment systemic and mucosal immune responses in mice against the native UspA2 outer membrane protein of *M. catarrhalis* was examined. Purified UspA2 (5 µg/dose) alone in 10 µl saline or in a 10 µl formulation containing 0.1 µg/dose of a mutant CT-CRM (CT-CRM_{E29H}, CT-CRM_{I16A}, CT-CRM_{I16A,S68Y}, CT-CRM_{V72Y} or CT-CRM_{S68Y,V72Y}) was administered IN at days 0, 7 and 14. Protein-specific IgG and IgA levels in the serum and in mucosal lavages were

examined at day 28. Statistically significant levels of IgG and IgA were detected only in the serum of animals immunized with CT-CRM_{E29H}, CT-CRM_{I16A,S68Y} and CT-CRM_{V72Y}. Significant levels of IgG were also detected in the bronchial wash of animals immunized with CT-CRM_{E29H}, CT-CRM_{I16A,S68Y} and CT-CRM_{V72Y}.

5 *B. Nucleic Acid Molecules Encoding CT-CRMs*

Another aspect of this invention includes isolated, synthetic or recombinant nucleic acid molecules and sequences encoding the above-described CT-CRMs having the specified site directed mutations or fragments that may further contain one or more of those mutations.

10 An isolated nucleotide molecule comprising a nucleic acid sequence encoding a CT-CRM protein may be preferably under the control of regulatory sequences that direct expression of the CT-CRM in a host cell. As described herein, such nucleic acid molecules may be used to express the CT-CRM protein *in vitro* or to permit expression of the CT-CRM protein *in vivo* in a human.

15 As used herein, the term "isolated nucleotide molecule or sequence" refers to a nucleic acid segment or fragment which is free from contamination with other biological components that may be associated with the molecule or sequence in its natural environment. For example, one embodiment of an isolated nucleotide molecule or sequence of this invention is a sequence separated from sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally adjacent to the fragment, such as the sequences adjacent to the fragment in a genome in which it naturally occurs. Further, the nucleotide sequences and molecules of this invention have been altered to encode a CT-CRM protein of this invention. Thus, the term "isolated nucleic acid molecule or sequence" 20 also applies to nucleic acid sequences or molecules that have been substantially purified from other components that naturally accompany the unmutagenized nucleic acid, e.g., RNA or DNA or proteins, in the cell. An isolated nucleotide molecule or sequence of this invention also encompasses sequence and molecules that have been prepared by other conventional methods, such as recombinant methods, synthetic methods, e.g., mutagenesis, or combinations of such methods. The nucleotide sequences or 25 molecules of this invention should not be construed as being limited solely to the 30

specific nucleotide sequences presented herein, but rather should be construed to include any and all nucleotide sequences which share homology (i.e., have sequence identity) with the nucleotide sequences presented herein.

The terms "substantial homology" or "substantial similarity," when referring to a nucleic acid or fragment thereof, indicates that, when optimally aligned with appropriate nucleotide insertions or deletions with another nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 70% of the nucleotide bases, as measured by any well-known algorithm of sequence identity, such as FASTA, a program in GCG Version 6.1. The term "homologous" as used herein, refers to the sequence similarity between two polymeric molecules, *e.g.*, between two nucleic acid molecules, *e.g.*, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a nucleotide or amino acid position in both of the two molecules is occupied by the same monomeric nucleotide or amino acid, *e.g.*, if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, *e.g.*, if half (*e.g.*, five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous. If 90% of the positions, *e.g.*, 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC5' and 3'TATGCG5' share 50% homology. By the term "substantially homologous" as used herein, is meant DNA or RNA which is about 70% homologous, more preferably about 80% homologous and most preferably about 90% homologous to the desired nucleic acid.

The invention is also directed to an isolated nucleotide molecule comprising a nucleic acid sequence that is at least 70%, 80% or 90% homologous to a nucleic acid sequence encoding a CT-CRM protein of this invention that has reduced enzymatic toxicity compared to wild-type CT protein and that retains adjuvanticity of the wild-type CT. Furthermore, due to the degeneracy of the genetic code, any three-nucleotide codon that encodes a mutated amino acid residue of CT-CRM, described herein is within the scope of the invention.

Where, as discussed herein, CT-CRMs, mutant CT-A subunits or mutant CT-B subunits, and/or DNA sequences encoding them, or other sequences useful in nucleic acid molecules or compositions described herein are defined by their percent homologies or identities to identified sequences, the algorithms used to calculate the percent homologies or percent identities include the following: the Smith-Waterman algorithm (J. F. Collins *et al*, 1988, *Comput. Appl. Biosci.*, 4:67-72; J. F. Collins *et al*, Molecular Sequence Comparison and Alignment, (M. J. Bishop *et al*, eds.) In Practical Approach Series: Nucleic Acid and Protein Sequence Analysis XVIII, IRL Press: Oxford, England, UK (1987) pp.417), and the BLAST and FASTA programs (E. G. Shpaer *et al*, 1996, *Genomics*, 38:179-191). These references are incorporated herein by reference.

By describing two DNAs as being "operably linked" as used herein, is meant that a single-stranded or double-stranded DNA comprises each of the two DNAs and that the two DNAs are arranged within the DNA in such a manner that at least one of the DNA sequences is able to exert a physiological effect by which it is characterized upon the other.

Preferably, for use in producing a CT-CRM protein of this invention or administering it for *in vivo* production in a cell, each CT-CRM protein encoding sequence and necessary regulatory sequences are present in a separate viral or non-viral recombinant vector (including non-viral methods of delivery of a nucleic acid molecule into a cell). Alternatively, two or more of these nucleic acid sequences encoding duplicate copies of a CT-CRM protein or encoding multiple different CT-CRMs of this invention may be contained in a polycistronic transcript, i.e., a single molecule designed to express multiple gene products.

The invention further relates to vectors, particularly plasmids, containing isolated and purified DNA sequences comprising DNA sequences that encode an immunogenic mutant cholera holotoxin. Desirable embodiments include plasmids containing DNA sequences encoding CT-CRMs having a single amino acid substitution at amino acid position 16 or 72 or double amino acid substitution at amino acid position 16 and 68 or 68 and 72 respectively. By the term "vector" as used herein, is meant a DNA molecule derived from viral or non-viral, e.g., bacterial,

species that has been designed to encode an exogenous or heterologous nucleic acid sequence. Thus, the term includes conventional bacterial plasmids. Such plasmids or vectors can include plasmid sequences from viruses or phages. Such vectors include chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial 5 plasmids, bacteriophages, yeast episomes, yeast chromosomal elements, and viruses. Vectors may also be derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, cosmids, and phagemids. The term also includes non-replicating viruses that transfer a gene from one cell to another. The term should also be construed to include non-plasmid and non-viral compounds which 10 facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds and the like.

The nucleic acid molecules of the invention include non-viral vectors or methods for delivery of the sequence encoding the CT-CRM protein to a host cell according to this invention. A variety of non-viral vectors are known in the art and 15 may include, without limitation, plasmids, bacterial vectors, bacteriophage vectors, "naked" DNA and DNA condensed with cationic lipids or polymers.

Examples of bacterial vectors include, but are not limited to, sequences derived from *bacille Calmette Guérin* (BCG), *Salmonella*, *Shigella*, *E. coli*, and *Listeria*, among others. Suitable plasmid vectors include, for example, pBR322, 20 pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pK37, pKC101, pAC105, pVA51, pKH47, pUB110, pMB9, pBR325, Col E1, pSC101, pBR313, pML21, RSF2124, pCR1, RP4, pBAD18, and pBR328.

Examples of suitable inducible *Escherichia coli* expression vectors include pTrc (Amann *et al.*, 1988 *Gene*, 69:301-315), the arabinose expression vectors (e.g., pBAD18, Guzman *et al.*, 1995 *J. Bacteriol.*, 177:4121-4130), and pETIId (Studier *et al.*, 1990 *Methods in Enzymology*, 185:60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion 25 promoter. Target gene expression from the pETIId vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase T7 gn1. This viral polymerase is supplied by host strains BL21 (DE3) or HMS I 74(DE3) 30 from a resident prophage harboring a *T7 gn1* gene under the transcriptional control of

the *lacUV5* promoter. The pBAD system relies on the inducible arabinose promoter that is regulated by the *araC* gene. The promoter is induced in the presence of arabinose.

As one example, a plasmid, designated pLP903, contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic mutant CT-CRM with substantially reduced toxicity wherein the amino acid alanine is substituted for isoleucine at amino acid position 16 in the A subunit. A second plasmid, designated pLP905, contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic mutant CT-CRM with substantially reduced toxicity wherein the amino acid tyrosine is substituted for valine at the amino acid position 72 in the A subunit. Another exemplary plasmid is designated pLP904. This plasmid contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic, mutant CT-CRM with substantially reduced toxicity wherein the amino acid alanine is substituted for isoleucine at amino acid position 16, and amino acid tyrosine is substituted for serine at amino acid position 68 in the A subunit. Another plasmid exemplified in this invention is designated pLP906. It contains an isolated and purified DNA sequence comprising a DNA sequence encoding an immunogenic, mutant CT-CRM with substantially reduced toxicity wherein the amino acid tyrosine is substituted for serine at the amino acid position 68, and amino acid tyrosine is substituted for valine at amino acid position 72 in the A subunit.

Another type of useful vector is a single or double-stranded bacteriophage vector. For example, a suitable cloning vector includes, but is not limited to the vectors such as bacteriophage λ vector system, λ gt11, μ gt μ WES.tB, Charon 4, λ gt-WES- λ B, Charon 28, Charon 4A, λ gt-1- λ BC, λ gt-1- λ B, M13mp7, M13mp8, or M13mp9, among others.

In another embodiment, the expression vector is a yeast expression vector. Examples of vectors for expression in a yeast such as *S. cerevisiae* include pYEpSec I (Baldari, *et al.*, 1987 *Protein Eng.*, 1(5):433-437), pMFA (Kurjan and Herskowitz, 1982 *Cell*, 30(3):933-943), pJRY88 (Schultz *et al.*, 1987 *Gene*, 61(2):123-133), and pYES2 (Invitrogen Corporation, San Diego, CA).

Alternatively, baculovirus expression vectors are used. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 or Sf 21 cells) include the pAc series (Smith *et al.*, 1983 *Biotechnol.*, 24:434-443) and the pVL series (Luckow and Summers, 1989 *Virol.*, 170(1):31-39). In yet another embodiment, a 5 mammalian expression vector is used for expression in mammalian cells. Examples of mammalian expression vectors include pCDM8 (Seed, 1987 *Nature*, 329:840-842) and pMT2PC (Kaufman *et al.*, 1987 *EMBO J.*, 6(1):187-93). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements.

10 One type of recombinant vector is a recombinant single or double-stranded RNA or DNA viral vector. A variety of viral vector systems are known in the art. Examples of such vectors include, without limitation, recombinant adenoviral vectors, herpes simplex virus (HSV)-based vectors, adeno-associated viral (AAV) vectors, hybrid adenoviral/AAV vectors, recombinant retroviruses or lentiviruses, recombinant 15 poxvirus vectors, recombinant vaccinia virus vectors, SV-40 vectors, insect viruses such as baculoviruses, and the like that are constructed to carry or express a selected nucleic acid composition of interest.

Retrovirus vectors that can be employed include those described in EP 0 415 731; International Patent Publication Nos. WO 90/07936; WO 94/03622; WO 20 93/25698; and WO 93/25234; U.S. Patent No. 5,219,740; International Patent Publication Nos. WO 93/11230 and WO 93/10218; Vile and Hart, 1993 *Cancer Res.* 53:3860-3864; Vile and Hart, 1993 *Cancer Res.* 53:962-967; Ram *et al.*, 1993 *Cancer Res.* 53:83-88; Takamiya *et al.*, 1992 *J. Neurosci. Res.* 33:493-503; Baba *et al.*, 1993 *J. Neurosurg.* 79:729-735; US Patent No. 4,777,127; GB Patent No. 2,200,651; and 25 EP 0 345 242. Examples of suitable recombinant retroviruses include those described in International Patent Publication No. WO 91/02805.

Alphavirus-based vectors may also be used as the nucleic acid molecule encoding the CT-CRM protein. Such vectors can be constructed from a wide variety of viruses, including, for example, Sindbis virus vectors, Semliki forest virus (ATCC 30 VR-67; ATCC VR-1247), Ross River virus (ATCC VR-373; ATCC VR-1246) and Venezuelan equine encephalitis virus (ATCC VR-923; ATCC VR-1250; ATCC VR

1249; ATCC VR-532). Representative examples of such vector systems include those described in U.S. Patent Nos. 5,091,309; 5,217,879; and 5,185,440; and International Patent Publication Nos. WO 92/10578; WO 94/21792; WO 95/27069; WO 95/27044; and WO 95/07994.

5 Examples of adenoviral vectors include those described by Berkner, 1988 *Biotechniques* 6:616-627; Rosenfeld *et al.*, 1991 *Science* 252:431-434; International Patent Publication No. WO 93/19191; Kolls *et al.*, 1994 *PNAS* 91:215-219; Kass-Eisler *et al.*, 1993 *PNAS* 90:11498-11502; Guzman *et al.*, 1993 *Circulation* 88:2838-2848; Guzman *et al.*, 1993 *Cir. Res.* 73:1202-1207; Zabner *et al.*, 1993 *Cell* 75:207-216; Li *et al.*, 1993 *Hum. Gene Ther.* 4:403-409; Cailaud *et al.*, 1993 *Eur. J. Neurosci.* 5:1287-1291; Vincent *et al.*, 1993 *Nat. Genet.* 5:130-134; Jaffe *et al.*, 1992 *Nat. Genet.* 1:372-378; and Levrero *et al.*, 1991 *Gene* 101:195-202. Exemplary adenoviral vectors include those described in International Patent Publication Nos. WO 94/12649; WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655. Other adenoviral vectors include those derived from chimpanzee adenoviruses, such as those described in US Patent No. 6,083,716.

Another viral vector is based on a parvovirus such as an adeno-associated virus (AAV). Representative examples include the AAV vectors described in International Patent Publication No. WO 93/09239, Samulski *et al.*, 1989 *J. Virol.* 63:3822-3828; Mendelson *et al.*, 1988 *Virol.* 166:154-165; and Flotte *et al.*, 1993 *PNAS* 90:10613-10617. Other particularly desirable AAV vectors include those based upon AAV1; see, International Patent Publication No. WO 00/28061, published May 18, 2000. Other desirable AAV vectors include those which are pseudotyped, i.e., contain a minigene composed of AAV 5' ITRS, a transgene, and AAV 3' ITRs packaged in a capsid of an AAV serotype heterologous to the AAV ITRs. Methods of producing such pseudotyped AAV vectors are described in detail in International Patent Publication No. WO01/83692.

In an embodiment in which the nucleic acid molecule of the invention is "naked DNA", it may be combined with polymers including traditional polymers and non-traditional polymers such as cyclodextrin-containing polymers and protective, interactive noncondensing polymers, among others. The "naked" DNA and DNA

condensed with cationic lipids or polymers are typically delivered to the cells using chemical methods. A number of chemical methods are known in the art for cell delivery and include using lipids, polymers, or proteins to complex with DNA, optionally condensing the same into particles, and delivering to the cells. Another non-viral chemical method includes using cations to condense DNA, which is then placed in a liposome and used according to the present invention. See, C. Henry, 2001 *Chemical and Engineering News*, 79(48):35-41.

The nucleic acid molecule encoding the CT-CRM of this invention is introduced directly into the cells either as "naked" DNA (U.S. Patent No. 5, 580,859) or formulated in compositions with agents, which facilitate immunization, such as bupivacaine and other local anesthetics (U.S. Patent No. 6,127,170).

All components of the viral and non-viral vectors above may be readily selected from among known materials in the art and available from the pharmaceutical industry. Selection of the vector components and regulatory sequences are not considered a limitation on this invention. Each nucleic acid sequence encoding a CT-CRM protein according to this invention is preferably under the control of regulatory sequences that direct the replication and generation of the product of each nucleic acid sequence in a mammalian or vertebrate cell. By the term "promoter/regulatory sequence" is meant a DNA sequence required for expression of a nucleic acid operably linked to the promoter/regulatory sequence. In some instances, the promoter/regulatory sequence may function in a tissue specific manner. For example, the promoter/regulatory sequence is only capable of driving expression in a cell of a particular tissue type. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements that are required for expression in a tissue-specific manner.

Preferably, the nucleic acid molecule encoding a CT-CRM protein of this invention and/or the recombinant vector further comprises regulatory sequences. For example, such regulatory sequences comprise a promoter that drives expression of the CT-CRM protein. Preferably the promoter/regulatory sequence is positioned at the 5' end of the coding sequence such that it drives expression of the CT-CRM protein in a cell.

Suitable promoters may be readily selected from among constitutive promoters, inducible promoters, tissue-specific promoters and others. Examples of constitutive promoters that are non-specific in activity and employed in the nucleic acid molecules encoding the CT-CRM protein of this invention include, without limitation, the retroviral Rous sarcoma virus (RSV) promoter, the retroviral LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) (see, e.g., Boshart et al, *Cell*, 41:521-530 (1985)), the SV40 promoter, the dihydrofolate reductase promoter, the β -actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1 α promoter (Invitrogen).

Inducible promoters that are regulated by exogenously supplied compounds, include, without limitation, the arabinose promoter, the zinc-inducible sheep metallothioneine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al, 1996 *Proc. Natl. Acad. Sci. USA*, 93:3346-3351), the tetracycline-repressible system (Gossen et al, 1992 *Proc. Natl. Acad. Sci. USA*, 89:5547-5551), the tetracycline-inducible system (Gossen et al, 1995 *Science*, 268:1766-1769, see also Harvey et al, 1998 *Curr. Opin. Chem. Biol.*, 2:512-518), the RU486-inducible system (Wang et al, 1997 *Nat. Biotech.*, 15:239-243 and Wang et al, 1997 *Gene Ther.*, 4:432-441) and the rapamycin-inducible system (Magari et al, 1997 *J. Clin. Invest.*, 100: 2865-2872). A particularly preferred promoter for use in expression systems for CT-CRMs is an arabinose inducible promoter.

Other types of inducible promoters that may be useful in this context are those regulated by a specific physiological state, e.g., temperature or acute phase or in replicating cells only. Useful tissue-specific promoters include the promoters from genes encoding skeletal β -actin, myosin light chain 2A, dystrophin, muscle creatine kinase, as well as synthetic muscle promoters with activities higher than naturally-occurring promoters (see Li et al., 1999 *Nat. Biotech.*, 17:241-245). Examples of promoters that are tissue-specific are known for liver (albumin, Miyatake et al. 1997 *J. Virol.*, 71:5124-32; hepatitis B virus core promoter, Sandig et al., 1996 *Gene Ther.*, 3: 1002-9; alpha-fetoprotein (AFP), Arbuthnot et al., 1996 *Hum. Gene Ther.*, 7:1503-14), bone (osteocalcin, Stein et al., 1997 *Mol. Biol. Rep.*, 24:185-96; bone

sialoprotein, Chen *et al.*, 1996 *J. Bone Miner. Res.*, 11:654-64), lymphocytes (CD2, Hansal *et al.*, 1988 *J. Immunol.*, 161:1063-8; immunoglobulin heavy chain; T cell receptor α chain), neuronal (neuron-specific enolase (NSE) promoter, Andersen *et al.* 1993 *Cell. Mol. Neurobiol.*, 13:503-15; neurofilament light-chain gene, Piccioli *et al.*, 5 1991 *Proc. Natl. Acad. Sci. USA*, 88:5611-5; the neuron-specific B ngf gene, Piccioli *et al.*, 1995 *Neuron*, 15:373-84); among others. See, e.g., International Patent Publication No. WO00/55335 for additional lists of known promoters useful in this context.

Additional regulatory sequences for inclusion in a nucleic acid sequence, 10 molecule or vector of this invention include, without limitation, an enhancer sequence, a polyadenylation sequence, a splice donor sequence and a splice acceptor sequence, a site for transcription initiation and termination positioned at the beginning and end, respectively, of the polypeptide to be translated, a ribosome binding site for translation in the transcribed region, an epitope tag, a nuclear localization sequence, an IRES element, a Goldberg-Hogness "TATA" element, a restriction enzyme cleavage site, a selectable marker and the like. Enhancer sequences include, e.g., the 72 bp tandem repeat of SV40 DNA or the retroviral long terminal repeats or LTRs, etc. and are employed to increase transcriptional efficiency. Selection of promoters and other common vector elements are conventional and many such sequences are available with 15 which to design the nucleotide molecules and vectors useful in this invention. See, e.g., Sambrook *et al*, *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, (1989) and references cited therein at, for example, pages 3.18-3.26 and 16.17-16.27 and Ausubel *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1989). One of skill in the art may readily 20 select from among such known regulatory sequences to prepare molecules of this invention. The selection of such regulatory sequences is not a limitation of this invention.

C. Methods for Making the CT-CRM Proteins and Nucleotide Molecules of this Invention

In view of the demonstrated utility of mutant CT-CRMs as adjuvants for 30 antigenic compositions, production of suitable quantities of mutant CT-CRMs is

desirable. The preparation or synthesis of the nucleotide sequences and CT-CRMs, as well as compositions containing the nucleotide molecules or CT-CRM protein of this invention disclosed herein is well within the ability of the person having ordinary skill in the art using available material. The synthesis methods are not a limitation of this 5 invention. The examples below detail presently preferred embodiments of synthesis of sequences encoding the CT-CRMs of this invention.

The CT-CRMs and nucleotide molecules and sequences of this invention may be produced by chemical synthesis methods, recombinant genetic engineering methods, site directed mutagenesis, among others, and combinations of such methods. For 10 example, the nucleotide sequences/CT-CRMs of the invention may be prepared conventionally by resort to known chemical synthesis techniques, e.g., solid-phase chemical synthesis, such as described by Merrifield, 1963 *J. Amer. Chem. Soc.*, 85:2149-2154; J. Stuart and J. Young, Solid Phase Peptide Synthesis, Pierce Chemical Company, Rockford, IL (1984); Matteucci *et al.*, 1981 *J. Am. Chem. Soc.*, 103:3185; 15 Alvarado-Urbina *et al.*, 1980 *Science*, 214:270; and Sinha, N. D. *et al.*, 1984 *Nucl. Acids Res.*, 13:4539, among others. See, also, e.g., PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993; Wold, F., "Posttranslational Protein Modifications: Perspectives and Prospects", pgs. 1-12 in POSTTRANSLATIONAL COVALENT 20 MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter *et al.*, 1990 *Meth. Enzymol.*, 182:626-646, and Rattan *et al.*, 1992 *Ann. N.Y. Acad. Sci.*, 663:48-62.

Alternatively, compositions of this invention may be constructed recombinantly using conventional molecular biology techniques, site-directed mutagenesis, genetic 25 engineering or polymerase chain reaction, such as, by cloning and expressing a nucleotide molecule encoding a CT-CRM protein with optional other immunogens and optional carrier proteins within a host microorganism, etc. utilizing the information provided herein (See, e.g., Sambrook *et al.*, Molecular Cloning. A Laboratory Manual, 2d Edit., Cold Spring Harbor Laboratory, New York (1989); Ausubel *et al.* Current 30 Protocols in Molecular Biology, John Wiley & Sons, New York (1997)). Coding

sequences for the CT-CRMs and optional immunogens can be prepared synthetically (W. P. C. Stemmer *et al*, 1995 *Gene*, 164:49).

In general, recombinant DNA techniques involve obtaining by synthesis or isolation a DNA sequence that encodes the CT-CRM protein as described above, and
5 introducing it into an appropriate vector/host cell expression system where it is expressed, preferably under the control of an arabinose inducible promoter. Any of the methods described for the insertion of DNA into an expression vector may be used to ligate a promoter and other regulatory control elements into specific sites within the selected recombinant vector. Suitable host cells are then transformed, infected,
10 transduced or transfected with such vectors or plasmids by conventional techniques.

A variety of host cell-vector (plasmid) systems may be used to express the immunogenic mutant cholera holotoxin. The vector system, which preferably includes the arabinose inducible promoter, is compatible with the host cell used. The DNA encoding the mutant CT-CRMs are inserted into an expression system, and the
15 promoter (preferably the arabinose inducible promoter), and other control elements are ligated into specific sites within the vector so that when the vector is inserted into a host cell (by transformation, transduction or transfection, depending on the host cell-vector system used) the DNA encoding the CT-CRM is expressed by the host cell.

The vector may be selected from one of the viral vectors or non-viral vectors
20 described above but must be compatible with the host cell used. The recombinant DNA vector may be introduced into appropriate host cells (bacteria, virus, yeast, mammalian cells or the like) by transformation, transduction or transfection (depending upon the vector/host cell system). Host-vector systems include but are not limited to bacteria transformed with bacteriophage DNA, plasmid DNA or cosmid DNA;
25 microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); and insect cell systems infected with virus (e.g., baculovirus).

Systems for cloning and expressing the CT-CRMs and other compositions of this invention using the synthetic nucleic acid molecules include the use of various
30 microorganisms and cells that are well known in recombinant technology. The host cell may be selected from any biological organism, including prokaryotic (e.g.,

bacterial) cells and eukaryotic cells, including, mammalian, insect cells, yeast cells. Preferably, the cells employed in the various methods and compositions of this invention are bacterial cells. Suitable bacterial cells include, for example, various strains of *E. coli*, *Bacillus*, and *Streptomyces*. Yeast cells such as *Saccharomyces* and 5 *Pichia*, and insect cells such as Sf9 and Sf21 cells are also useful host cells for production purposes. Mammalian cells including but not limited to Chinese hamster ovary cells (CHO), chick embryo fibroblasts, baby hamster kidney cells, NIH3T3, PER C6, NSO, VERO or COS cells are also suitable host cells, as well as other conventional and non-conventional organisms and plants.

10 The selection of other suitable host cells and methods for transformation, culture, amplification, screening and product production and purification can be performed by one of skill in the art by reference to known techniques. See, e.g., Gething and Sambrook, 1981 *Nature*, 293:620-625, among others.

15 Typically, the host cell is maintained under culture conditions for a period of time sufficient for expression. Culture conditions are well known in the art and include ionic composition and concentration, temperature, pH and the like. Typically, transfected cells are maintained under culture conditions in a culture medium. Suitable media for various cell types are well known in the art. In a preferred embodiment, temperature is from about 20°C to about 50°C, more preferably from about 30°C to 20 about 40°C and, even more preferably about 37°C.

The pH is preferably from about a value of 6.0 to a value of about 8.0, more 25 preferably from about a value of about 6.8 to a value of about 7.8 and, most preferably about 7.4. Osmolality is preferably from about 200 milliosmols per liter (mosm/L) to about 400 mosm/l and, more preferably from about 290 mosm/L to about 310 mosm/L. Other biological conditions needed for transfection and expression of an encoded protein are well known in the art.

Recombinant CT-CRM protein is recovered or collected either from the host 30 cells or membranes thereof or from the medium in which those cells are cultured. Recovery comprises isolating and purifying the recombinant CT-CRM protein. Isolation and purification techniques for polypeptides are well known in the art and

include such procedures as precipitation, filtration, chromatography, electrophoresis and the like.

When produced by conventional recombinant means, CT-CRMs of this invention may be isolated and purified from the cell or medium thereof by conventional methods, including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard techniques for the purification of proteins. Several techniques exist for purification of heterologous protein from prokaryotic cells. See, US Patent Nos. 4,518,526; 4,599,197; and 4,734,362. The purified preparation however produced should be substantially free of host toxins, which might be harmful to humans. In particular, when expressed in gram negative bacterial host cells such as *E. coli*, the purified peptide or protein should be substantially free of endotoxin contamination. See, e.g., Sambrook *et al.*, Molecular Cloning. A Laboratory Manual., 2d Edit., Cold Spring Harbor Laboratory, New York (1989).

The CT-CRMs used in methods and compositions of the invention are not limited to products of any of the specific exemplary processes listed herein. In fact, the protein may be prepared by the methods in the texts cited immediately above or by methods of the texts cited elsewhere in this specification. It is within the skill of the art to isolate and produce recombinantly or synthetically protein compositions for such use.

The four exemplary CT-CRMs of Table 1, two bearing a single amino acid substitution and two bearing double amino acid substitutions were generated as described in detail in Example 1 using some of the methods described above. Specifically, a set of mutant CT clones (CT-CRMs) were generated in *E. coli* by standard site-directed mutagenesis protocols on plasmids encoding the known CT holotoxin molecules.

It has previously been shown that the resulting yield of purified CT-CRM_{E29H} holotoxin was approximately 50 µg per liter of culture medium (see International patent publication No. WO 00/18434). Initial attempts to increase CT-CRM_{E29H} yield via modifications to the original plasmid, showed little or no effect. A moderate increase in yield was achieved through co-expression of the plasmid pIIB29H, and

derivatives, with *Vibrio cholerae* DsbA and *E. coli* RpoH. Co-expression and purification modifications increased the yield of CT-CRM_{E29H} to approximately 2 mg/liter.

In order to increase the expression of CT-CRMs of the present invention, the lactose inducible promoter in the plasmids was replaced with an arabinose inducible promoter (Invitrogen Corporation, Carlsbad, CA), which was operatively linked to the DNA sequence encoding the CT-CRMs. During cloning it was determined that plasmid pIIB29H contained a *ctxA* gene encoding CT subunit A from *Vibrio cholerae* strain 569B, linked to a *ctxB* gene encoding CT subunit B from *Vibrio cholerae* strain 2125. Cross alignment of these genes indicated seven base substitutions between the two *ctxB* genes and a single base change between the *ctxA* genes. Several of these base substitutions led to amino acid changes in the mature subunits. Of special note is the substitution between the *ctxA* genes which leads to an amino acid change within the A-2 portion, or the holotoxin assembly domain of the A subunit. It was not known whether the heterogeneity between these genes had a negative impact on toxin expression or holotoxin assembly. However, it was thought preferable from an evolutionary standpoint that both toxin subunit genes originate from the same source. As such, both the *ctxA* and *ctxB* genes used in the construction of the arabinose inducible system originated from *Vibrio cholerae* strain 569B. The construction of plasmids pLP903, pLP904, pLP905, pLP906, is described in Example 1. The immunogenic mutant cholera holotoxin is produced by transforming, infecting, transducing or transfecting a host cell with a plasmid described above, and culturing the host cell under conditions that permit the expression of said recombinant immunogenic detoxified protein by the host cell. Production of CT-CRMs from pLP903, pLP904, pLP905 and pLP906 is approximately 10mg of purified material per liter of culture.

The resulting CT-CRM protein or nucleic acid molecule may be formulated into an immunogenic composition with any number of selected antigens and screened for adjuvant efficacy by *in vivo* assays, such as those described in the examples below.

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D. Immunogenic Compositions

An effective immunogenic composition according to the invention is one comprising a mutant cholera holotoxin of this invention. Preferably the mutant cholera holotoxin CT-CRM has reduced toxicity compared to a wild-type cholera holotoxin.

5 This “reduced toxicity” enables each mutant to be used as an adjuvant in an immunogenic composition without causing significant side effects, particularly those known to be associated with wild-type CT, e.g., diarrhea. More preferably, the CT-CRM in the immunogenic composition of this invention has a single amino acid substitution at the amino acid position 16 or 72 in the A subunit of the holotoxin, or a double amino acid substitution at amino acid positions 16 and 68 or 68 and 72 of the A subunit of the cholera holotoxin. In one embodiment, the CT-CRM may have one or more additional modifications as described above. In another embodiment, the composition comprises a selected antigen and a suitable effective adjuvanting amount of the CT-CRM, wherein said holotoxin significantly enhances the immune response in a vertebrate host to said antigen. The compositions of the present invention modulate the immune response by improving the vertebrate host’s antibody response and cell-mediated immune responses to the administration of a composition comprising a selected antigen as described above.

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As used herein, the term “effective adjuvanting amount” means a dose of one of the CT-CRM mutants of this invention that is effective in eliciting an increased immune response in a vertebrate host. In a more specific definition, the term “effective adjuvanting amount” means a dose of one of the four CT-CRM mutants described herein (CT-CRM_{I16A}, CT-CRM_{V72Y}, CT-CRM_{I16A, S68Y}, CT-CRM_{S68Y, V72Y}), effective in eliciting an increased immune response in a vertebrate host. Specifically, the CT-CRMs disclosed herein augment mucosal and systemic immune responses following intranasal administration of disparate antigens. Furthermore, even in the presence of pre-existing anti-CT immune responses, the mutant CT-CRMs were able to serve as efficient mucosal adjuvants. The immunogenic mutant CT-CRMs according to the present invention exhibit a balance of reduced toxicity and retained adjuvanticity, such that the resulting mutant CT protein functions as an adjuvant while being tolerated safely by the vertebrate host to which it is introduced. The particular “effective

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adjuvanting dosage or amount" will depend upon the age, weight and medical condition of the host, as well as on the method of administration. Suitable doses are readily determined by persons skilled in the art.

The immunogenic compositions containing as an adjuvant the mutant cholera holotoxins of this invention also contain at least one antigen selected from among a wide variety of antigens. The antigen(s) may comprise a whole cell or virus, or one or more saccharides, proteins, protein subunits, polypeptide, peptide or fragments, poly- or oligonucleotides, or other macromolecular components. If desired, the antigenic compositions may contain more than one antigen from the same or different pathogenic microorganisms.

Thus, in one embodiment, the immunogenic compositions of this invention comprise as the selected antigen a polypeptide, peptide or fragment derived from a pathogenic bacterium. Desirable bacterial immunogenic compositions including the CT-CRM mutants as an adjuvant include those directed to the prevention and/or treatment of disease(s) caused by, without limitation, *Haemophilus influenzae* (both typable and nontypable), *Haemophilus somnus*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus faecalis*, *Helicobacter pylori*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Bordetella pertussis*, *Alloiococcus otiditis*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella choleraesuis*, *Escherichia coli*, *Shigella*, *Vibrio cholerae*, *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, *Mycobacterium avium-Mycobacterium intracellulare complex*, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Clostridium tetani*, *Leptospira interrogans*, *Borrelia burgdorferi*, *Pasteurella haemolytica*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* and *Mycoplasma gallisepticum*.

In another embodiment, the immunogenic compositions of this invention comprise as the selected antigen a polypeptide, peptide or fragment derived from a pathogenic virus. Desirable viral immunogenic compositions including the CT-CRM mutants as an adjuvant include those directed to the prevention and/or treatment of disease caused by, without limitation, Respiratory syncytial virus, Parainfluenza virus

types 1-3, Human metapneumovirus, Influenza virus, Herpes simplex virus, Human cytomegalovirus, Human immunodeficiency virus, Simian immunodeficiency virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Human papillomavirus, Poliovirus, rotavirus, caliciviruses, Measles virus, Mumps virus, Rubella virus,
5 adenovirus, rabies virus, canine distemper virus, rinderpest virus, avian pneumovirus (formerly turkey rhinotracheitis virus), Hendra virus, Nipah virus, coronavirus, parvovirus, infectious rhinotracheitis viruses, feline leukemia virus, feline infectious peritonitis virus, avian infectious bursal disease virus, Newcastle disease virus, Marek's disease virus, porcine respiratory and reproductive syndrome virus, equine arteritis
10 virus and various Encephalitis viruses.

In another embodiment, the immunogenic compositions of this invention comprise as the selected antigen a polypeptide, peptide or fragment derived from a pathogenic fungus. Desirable immunogenic compositions against fungal pathogens including the CT-CRM mutants as an adjuvant include those directed to the prevention and/or treatment of disease(s) caused by, without limitation, *Aspergillus*, *Blastomyces*,
15 *Candida*, *Coccidioides*, *Cryptococcus* and *Histoplasma*.

In still another embodiment, the immunogenic compositions of this invention comprise as the selected antigen a polypeptide, peptide or fragment derived from a pathogenic parasite. Desirable immunogenic compositions against parasites including the CT-CRM mutants as an adjuvant include those directed to the prevention and/or treatment of disease(s) caused by, without limitation, *Leishmania major*, *Ascaris*,
20 *Trichuris*, *Giardia*, *Schistosoma*, *Cryptosporidium*, *Trichomonas*, *Toxoplasma gondii* and *Pneumocystis carinii*.

Desirable immunogenic compositions directed against non-infectious diseases including the CT-CRM mutants as an adjuvant are also within the scope of this invention. Such immunogenic compositions include those directed to vertebrate antigens, particularly compositions directed against antigens for the prevention and/or treatment of disease(s), without limitation, such as allergy, autoimmune disease, Alzheimer disease and cancer.
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For example, the immunogenic composition of this invention may contain a polypeptide, peptide or fragment derived from a cancer cell or tumor cell. Desirable
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immunogenic compositions for eliciting a therapeutic or prophylactic anti-cancer effect in a vertebrate host, which contain the CT-CRM mutants of this invention, include those utilizing a cancer antigen or tumor-associated antigen including, without limitation, prostate specific antigen, carcino-embryonic antigen, MUC-1, Her2, CA-5 125, MAGE-3, hormones, hormone analogs and so forth.

Other immunogenic compositions of this invention are desirable for moderating responses to allergens in a vertebrate host. Such compositions contain the CT-CRM mutant(s) of this invention and a polypeptide, peptide or fragment derived from an allergen or fragment thereof. Examples of such allergens are described in the United 10 States Patent No. 5,830,877 and International patent publication No. WO 99/51259, which are hereby incorporated by reference, and include pollen, insect venoms, animal dander, fungal spores and drugs (such as penicillin). The immunogenic compositions interfere with the production of IgE antibodies, a known cause of allergic reactions, so as to moderate allergic responses to the allergen.

In still another embodiment, the immunogenic compositions of this invention contain as the selected antigen a polypeptide, peptide or fragment derived from a molecular portion of an antigen, which represents those produced by a host (a self molecule) in an undesired manner, amount or location, such as those from amyloid precursor protein so as to prevent or treat disease characterized by amyloid deposition 15 in a vertebrate host. Desirable compositions for moderating responses to self molecules in a vertebrate host, which contain CT-CRM mutants of this invention, include those containing a self molecule or fragment thereof. Examples of such self molecules include β -chain insulin involved in diabetes, the G17 molecule involved in 20 gastroesophageal reflux disease, and antigens which downregulate autoimmune responses in diseases such as multiple sclerosis, lupus and rheumatoid arthritis.

Still other immunogenic compositions of this invention are desirable for preventing or treating disease characterized by amyloid deposition in a vertebrate host. Such compositions contain the CT-CRM mutant(s) of this invention as well as portions 25 of amyloid precursor protein (APP). This disease is referred to variously as Alzheimer's disease, amyloidosis or amyloidogenic disease. The β -amyloid peptide (also referred to as A β peptide) is a 42 amino acid fragment of APP, which is

generated by processing of APP by the β and γ secretase enzymes, and has the following sequence: Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala (SEQ ID NO: 3). In some patients, the amyloid deposit 5 takes the form of an aggregated A β peptide. Surprisingly, it has now been found that administration of isolated A β peptide induces an immune response against the A β peptide component of an amyloid deposit in a vertebrate host (International patent publication No. WO 99/27944). Thus, embodiments of this invention include the CT-CRM mutants of this invention plus A β peptide, as well as fragments of A β peptide 10 and antibodies to A β peptides or fragments thereof. One such fragment of A β peptide is the 28 amino acid peptide having the following sequence (U.S. Patent No. 4,666,829): Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys (SEQ ID NO: 4).

Such immunogenic compositions further comprise an immunologically acceptable diluent or a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. The antigenic compositions may also be mixed with such diluents or carriers in a conventional manner. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption 15 delaying agents, and the like, compatible with administration to humans or other vertebrate hosts. The appropriate carrier will be evident to those skilled in the art and will depend in large part upon the route of administration.

The immunogenic compositions may also include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable 25 sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (*i.e.*, powder or granular) form for reconstitution with a suitable vehicle (*e.g.*, sterile pyrogen-free water) prior to 30 parenteral administration of the reconstituted composition. Other parenterally-administrable formulations, which are useful, include those, which comprise the active

ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer system. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

Still additional components that may be present in the protein immunogenic compositions of this invention are adjuvants in addition to the CT-CRMs, preservatives, chemical stabilizers, or other antigenic proteins. Typically, stabilizers, adjuvants, and preservatives are optimized to determine the best formulation for efficacy in the target human or animal. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable stabilizing ingredients that may be used include, for example, casamino acids, sucrose, gelatin, phenol red, N-Z amine, monopotassium diphosphate, lactose, lactalbumin hydrolysate, and dried milk.

The antigenic compositions of this invention may comprise further adjuvants in addition to the mutant CT-CRMs. A conventional non-CT-CRM adjuvant used to enhance an immune response include, without limitation, MPLTM (3-O-deacylated monophosphoryl lipid A; Corixa, Hamilton, MT), which is described in U.S. Patent No. 4,912,094, which is hereby incorporated by reference. Also suitable for use as adjuvants are synthetic lipid A analogs or aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa (Hamilton, MT), and which are described in United States Patent No. 6,113,918, which is hereby incorporated by reference. One such AGP is 2-[(R)-3-Tetradecanoyloxytetradecanoylamino] ethyl 2-Deoxy-4-O-phosphono-3-O-[(R)-3-tetradecanoyloxytetradecanoyl]-2-[(R)-3-tetradecanoyloxytetradecanoyl-amino]-b-D-glucopyranoside, which is also known as 529 (formerly known as RC529). This 529 adjuvant is formulated as an aqueous form or as a stable emulsion.

Still other non-CT-CRM adjuvants include mineral oil and water emulsions, aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, etc., Amphigen, Avridine, L121/squalene, D-lactide-polylactide/glycoside, pluronic polyols,

muramyl dipeptide, killed *Bordetella*, saponins, such as Stimulon™ QS-21 (Antigenics, Framingham, MA.), described in U.S. Patent No. 5,057,540, which is hereby incorporated by reference, and particles generated therefrom such as ISCOMS (immunostimulating complexes), *Mycobacterium tuberculosis*, bacterial lipopolysaccharides, synthetic polynucleotides such as oligonucleotides containing a CpG motif (U.S. Patent No. 6,207,646, which is hereby incorporated by reference), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63, LT-R72, CT-S109, PT-K9/G129; see, e.g., International Patent Publication Nos. WO 93/13302 and WO 92/19265, incorporated herein by reference.

Various cytokines and lymphokines are also suitable for inclusion in the immunogenic compositions of this invention. One such cytokine is granulocyte-macrophage colony stimulating factor (GM-CSF), which has a nucleotide sequence as described in U.S. Patent No. 5,078,996, which is hereby incorporated by reference. A plasmid containing GM-CSF cDNA has been transformed into *E. coli* and has been deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, under Accession No. 39900. The cytokine Interleukin-12 (IL-12) is another adjuvant that is described in U.S. Patent No. 5,723,127, which is hereby incorporated by reference (available from Genetics Institute, Inc., Cambridge, MA). Other cytokines or lymphokines have been shown to have immune modulating activity, including, but not limited to, the interleukins 1- α , 1- β , 2, 4, 5, 6, 7, 8, 10, 13, 14, 15, 16, 17 and 18, the interferons- α , β and γ , granulocyte colony stimulating factor, and the tumor necrosis factors α and β , and are suitable for use as adjuvants.

Still other suitable optional components of the immunogenic compositions of this invention include, but are not limited to: surface active substances (e.g., hexadecylamine, octadecylamine, octadecyl amino acid esters, lysolecithin, dimethyl-dioctadecylammonium bromide), methoxyhexadecylglycerol, and pluronic polyols; polyamines, e.g., pyran, dextran sulfate, poly IC, carbopol; peptides, e.g., muramyl dipeptide, dimethylglycine, tuftsin; oil emulsions; and mineral gels, e.g., aluminum phosphate, etc. and immune stimulating complexes. The CT-CRM and antigen may

also be incorporated into liposomes, or conjugated to polysaccharides, lipopolysaccharides and/or other polymers for use in an immunogenic composition.

Immunogenic compositions of this invention including the CT-CRM mutant(s), or DNA sequences and molecules encoding the desired CT-CRM of this invention, are also useful as polynucleotide compositions (also known as DNA immunogenic compositions) or administered with polynucleotides encoding the selected antigen. For example, it has been previously demonstrated that BALB/c mice administered a formulation of plasmid DNA (pDNA) encoding the full length glycoprotein D of herpes simplex virus (HSV) type 2 (gD2), along with CT-CRM_{E29H} by the intradermal route generated a higher average cellular response than those that received plasmid DNA encoding HSV gD2 by itself by the intradermal route. In addition, the average serum antibody titers for mice, which received the plasmid DNA HSV gD2 composition along with CT-CRM_{E29H} was approximately the same as that seen in mice that received the plasmid DNA HSV gD2 composition without adjuvant. Similarly, the plasmid DNA HSV gD2 composition adjuvanted with CT-CRM_{E29H} also generated a gD2-specific antibody response in vaginal wash samples at levels that were comparable to those seen following the delivery of the non-adjuvanted composition by intradermal or intramuscular routes. Mice immunized with the plasmid DNA HSV gD2 composition adjuvanted with CT-CRM_{E29H} or CT and delivered by the intradermal route also generated substantially higher levels of gamma interferon and IL-5 than mice that received the plasmid DNA HSV-gD2 composition without adjuvant. Thus, the CT-CRMs enhance proliferative and gamma interferon responses when administered with a plasmid DNA composition against HSV.

In addition to a carrier as described above, immunogenic compositions composed of polynucleotide molecules desirably contain optional polynucleotide facilitating agents or "co-agents", such as a local anesthetic, a peptide, a lipid including cationic lipids, a liposome or lipidic particle, a polycation such as polylysine, a branched, three-dimensional polycation such as a dendrimer, a carbohydrate, a cationic amphiphile, a detergent, a benzylammonium surfactant, or another compound that facilitates polynucleotide transfer to cells. Such a facilitating agent includes bupivacaine (*see* U.S. Patent No. 5,593,972, which is hereby incorporated by

reference). Other non-exclusive examples of such facilitating agents or co-agents useful in this invention are described in U. S. Patent Nos. 5,703,055; 5,739,118; 5,837,533; International Patent Publication No. WO96/10038, published April 4, 1996; and International Patent Publication No WO94/16737, published August 8, 1994, which are each incorporated herein by reference.

Most preferably, the local anesthetic is present in an amount that forms one or more complexes with the nucleic acid molecules. When the local anesthetic is mixed with nucleic acid molecules or plasmids of this invention, it forms a variety of small complexes or particles that pack the DNA and are homogeneous. Thus, in one embodiment of the immunogenic compositions of this invention, the complexes are formed by mixing the local anesthetic and at least one plasmid of this invention. Any single complex resulting from this mixture may contain a variety of combinations of the different plasmids. Alternatively, in another embodiment of the compositions of this invention, the local anesthetic may be pre-mixed with each plasmid separately, and then the separate mixtures combined in a single composition to ensure the desired ratio of the plasmids is present in a single immunogenic composition, if all plasmids are to be administered in a single bolus administration. Alternatively, the local anesthetic and each plasmid may be mixed separately and administered separately to obtain the desired ratio. Where, hereafter, the term "complex" or "one or more complexes" or "complexes" is used to define this embodiment of the immunogenic composition, it is understood that the term encompasses one or more complexes with each complex containing a mixture of the CT-CRM-encoding plasmids and antigen-encoding plasmids, or a mixture of complexes formed discretely, wherein each complex contains only one type of plasmid, or a one or a mixture of complexes wherein each complex contains a polycistronic DNA. Preferably, the complexes are between about 50 to about 150 nm in diameter. When the facilitating agent used is a local anesthetic, preferably bupivacaine, an amount of from about 0.1 weight percent to about 1.0 weight percent based on the total weight of the polynucleotide composition is preferred. See, also, International Patent Publication No. WO99/21591, which is hereby incorporated by reference, and which teaches the incorporation of benzylammonium surfactants as co-agents, preferably administered in an amount of

between about 0.001-0.03 weight %. According to the present invention, the amount of local anesthetic is present in a ratio to said nucleic acid molecules of 0.01-2.5% w/v local anesthetic to 1-10 µg/ml nucleic acid. Another such range is 0.05-1.25% w/v local anesthetic to 100 µg/ml to 1 ml/ml nucleic acid.

5 As used, such a polynucleotide immunogenic composition expresses the CT-CRM and antigens on a transient basis *in vivo*; no genetic material is inserted or integrated into the chromosomes of the host. This use is thus distinguished from gene therapy, where the goal is to insert or integrate the genetic material of interest into the chromosome. An assay is used to confirm that the polynucleotides administered by
10 immunization do not rise to a transformed phenotype in the host (U.S. Patent No. 6,168,918).

The immunogenic compositions may also contain other additives suitable for the selected mode of administration of the composition. The composition of the invention may also involve lyophilized polynucleotides, which can be used with other
15 pharmaceutically acceptable excipients for developing powder, liquid or suspension dosage forms. See, e.g., Remington: The Science and Practice of Pharmacy, Vol. 2, 19th edition (1995), e.g., Chapter 95 Aerosols; and International Patent Publication No. WO99/45966, the teachings of which are hereby incorporated by reference. Routes of administration for these compositions may be combined, if desired, or adjusted.
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These nucleic acid molecule-containing immunogenic compositions can contain additives suitable for administration via any conventional route of administration. In some preferred embodiments, the immunogenic composition of the invention is prepared for administration to human subjects in the form of, for example, liquids, powders, aerosols, tablets, capsules, enteric-coated tablets or capsules, or
25 suppositories.

The immunogenic compositions of the present invention (whether protein-containing or nucleic acid molecule-containing compositions), as described above, are not limited by the selection of the conventional, physiologically acceptable, carriers, adjuvants, or other ingredients useful in pharmaceutical preparations of the types
30 described above. The preparation of these pharmaceutically acceptable compositions,

from the above-described components, having appropriate pH isotonicity, stability and other conventional characteristics is within the skill of the art.

E. Methods of Use of the Compositions of this Invention

The immunogenic compositions of this invention that comprise the CT-CRM alone or a combination of the CT-CRM and a selected antigen, are administered to a human or to a non-human vertebrate by a variety of routes to enhance the immune response to an antigen, preferably a disease-causing antigen, as identified above. The compositions of the present invention modulate the immune response by improving the vertebrate host's antibody response and cell-mediated immunity after administration of a composition comprising a selected antigen as described above, and an effective adjuvanting amount of a mutant CT-CRM, where the mutant CT-CRM has substantially reduced toxicity compared to a wild-type CT, and wherein the reduced toxicity is a result of a single amino acid substitution, a double amino acid substitution, or amino acid insertions.

In one embodiment, the immunogenic composition containing the CT-CRM (either as a protein or encoded by a nucleic acid molecule) is administered prior to administration of a composition comprising the selected antigen (either as a protein or as a nucleic acid). In another embodiment, the immunogenic composition is administered simultaneously with the antigen, whether it is administered in a composition containing both antigen and CT-CRM or as a separate composition from that of the antigen-containing composition. In still a further embodiment, the composition containing the CT-CRM is administered after the composition containing the antigen. It is preferable, although not required, that the antigen and the mutant CT-CRM be administered at the same time.

The immunogenic composition containing the CT-CRM may be administered as a protein or as a nucleic acid molecule encoding the protein, as described above. The immunogenic composition containing the CT-CRM may be administered as a protein in combination with a selected antigen administered as a protein. Alternatively, as described above, the CT-CRM immunogenic composition may be administered as a protein with a nucleic acid molecule encoding the antigen, as described above. Still

another alternative involves administering both the CT-CRM and the antigen as nucleic acid sequences encoding these proteins.

Any suitable route of administration may be employed to administer the immunogenic composition containing the CT-CRM. The route may be the same or different from a route selected to administer a composition containing the selected antigen, if the CT-CRM and antigen are administered in separate compositions or in different forms, e.g., protein or nucleic acids. Suitable routes of administration include, but are not limited to, intranasal, oral, vaginal, rectal, parenteral, intradermal, transdermal (*see, e.g.*, International patent publication No. WO 98/20734, which is hereby incorporated by reference), intramuscular, intraperitoneal, subcutaneous, intravenous and intraarterial. The appropriate route is selected depending on the nature of the immunogenic composition used, and an evaluation of the age, weight, sex and general health of the patient and the antigens present in the immunogenic composition, and similar factors by an attending physician.

In general, selection of the appropriate "effective amount" or dosage for the the CT-CRM and/or antigen components of the immunogenic composition(s) of the present invention will also be based upon the protein or nucleic acid form of the CT-CRM and antigen, the identity of the antigen in the immunogenic composition(s) employed, as well as the physical condition of the subject, most especially including the general health, age and weight of the immunized subject. The method and routes of administration and the presence of additional components in the immunogenic compositions may also affect the dosages and amounts of the CT-CRM and antigen. Such selection and upward or downward adjustment of the effective dose is within the skill of the art. The amount of CT-CRM and antigen required to induce an immune response, preferably a protective response, or produce an exogenous effect in the patient without significant adverse side effects varies depending upon these factors. Suitable doses are readily determined by persons skilled in the art.

As an example, in one embodiment, for the compositions containing protein components, e.g., a CT-CRM variant protein and/or antigen as described above, each dose may comprise between about 1 µg to about 20 mg of the protein per mL of a

sterile solution. Other dosage ranges may also be contemplated by one of skill in the art. Initial doses may be optionally followed by repeated boosts, where desirable. In another example, the amounts of nucleotide molecules in the DNA and vector compositions may be selected and adjusted by one of skill in the art. In one embodiment, each dose will comprise between about 50 µg to about 1 mg of CT-CRM-encoding or antigen-encoding nucleic acid, e.g., DNA plasmid, per mL of a sterile solution.

The number of doses and the dosage regimen for the composition are also readily determined by persons skilled in the art. Protection may be conferred by a single dose of the immunogenic composition containing the CT-CRM, or may require the administration of several doses with or without the selected antigen, in addition to booster doses at later times to maintain protection. In some instances, the adjuvant property of the mutant CT-CRM may reduce the number of doses containing antigen that are needed or may reduce the time course of the dosage regimen. The levels of immunity can be monitored to determine the need, if any, for boosters.

In order that this invention may be better understood, the following examples are set forth. The examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention.

All references cited herein are hereby incorporated by reference.

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EXAMPLE 1: EXPRESSION OF CT MUTANTS

A. Bacterial strains, plasmids and growth conditions

E. coli TG1 (Amersham-Pharmacia Biotech, Piscataway, NJ), and TX1, a nalidixic acid-resistant derivative of TG1, carrying FTc, lacIq from XL1 blue (Stratagene, LaJolla, CA; and CJ236(FTc, lacIq) (Bio-Rad, Hercules, CA) were used as hosts for cloning recombinant plasmids and expression of mutated proteins. Plasmid-containing strains were maintained on LB agar plates with antibiotics as required (ampicillin, 50 µg/ml; kanamycin 25 µg/ml; tetracycline 10 µg/ml). A complete CT operon from *V. cholerae* 0395 was subcloned into the phagemid vector pSKII-, under the control of the *lac* promoter, to create the IPTG inducible plasmid

designated pMGJ67 (Jobling, M.G., and Holmes, R.K., 1992 *Infect. Immun.*, 60, 4915-4924).

B. Mutagenesis of ctxA gene

The method of Kunkel, T.A., 1985 *Proc. Natl. Acad. Sci., USA*, 82, 488-492 was used to select for oligonucleotide-derived mutants created in plasmid pMGJ67. The oligonucleotides used to generate the mutant CT-CRMs and the various amino acid substitutions in the mutant CT-CRMs are listed in Table 2.

Table 2: Sequence of Oligonucleotides Introduced into ctxA

Substitution	Oligonucleotide Sequence ^a	SEQ ID NO.
I16A	CCTCCTGATGAAG <u>S</u> YCAAGCAGTCAGG	5
S68Y	GT <u>TTGAGAT</u> CTGCCCACT	6
V72Y	GT <u>TTGACCCACTAAGTGGGC</u>	7
S68Y+V72Y	GT <u>TTGAGAT</u> ATGCCCACT <u>TATG</u> GTCAAC	8

^a Altered bases are underlined. S represents G or C; Y represents C or T.

Briefly, the CT-CRM_{I16A} mutant was made directly in pMGJ142 using the QuickChange mutagenesis kit as described by the supplier (Stratagene Inc., LaJolla, CA). The double mutant plasmid containing the CT-CRM_{S68Y, V72Y} substitutions was made by PCR using the mutagenic primer disclosed in Table 2 to create a megaprimer followed by cloning of the mutated ctxA-encoding XbaI-ClaI fragment into pMGJ142. The CT-CRM_{I16A, S68Y} double mutant was made by PCR of the I16A containing clone using the mutagenic primer to create a megaprimer followed by cloning of the mutated ctxA-encoding XbaI-ClaI fragment into pMGJ142. The CT-CRM_{V72Y} and CT-CRM_{I16A, S68Y} mutants were made by reversion of the CT-CRM_{S68Y, V72Y} double mutant back to wild-type at amino acid position 68 using the QuickChange mutagenesis kit. Each single-stranded oligonucleotide was phosphorylated and used to direct second strand synthesis on a uracil-containing single-stranded DNA template rescued from the *E. coli dut ung* strain CJ236 (F'Tc, pMGJ67). Following ligation and transformation of *ung*⁺ strain TX1, single-stranded DNA was rescued from Amp^R transformants and sequenced by the dideoxy chain termination method (Kunkel, cited above).

C. *Construction of Arabinose Promoted CT-CRM Expression Vectors.*

Previous experience with CT-CRM_{E29H} (International patent publication No. WO 00/18434) has shown that maximal production in *E. coli* could be achieved by substituting synthetic Shine-Delgarno sequences upstream of the *ctxA* gene and placing the operon under the control of the arabinose promoter system. CT operons containing site directed mutations in the A subunit were made as previously described (*supra*).
5 CT-CRMs were originally under the control of a β lac promoter and expression levels in *E. coli* were low. PCR was used to modify the region 5' to the ATG of the CT-A subunit and insert an *NheI* site at the 5' end. The corresponding 3' primer added a
10 *HindIII* site at the 3' end of the CT-B gene.

Primer sequences used were:

CT forward: 5' TTTTTTGGGCTAGCATGGAGGAAAAGATGAGC

(SEQ. ID NO. 9); and

CT reverse: 5' CGAGGTCGAAGCTGCATGTTGGC (SEQ.ID NO. 10).

15 PCR was performed on each mutant CT-CRM operon and the PCR products were ligated into pCR2.1-Topo (Invitrogen) according to the manufacturer's directions and transformed into Top10F' cells. Recombinant *E. coli* were plated onto SOB agar containing Kanamycin (25 μ g/ml) and X-gal (40 μ g/ml). Plasmids from white colonies were screened for inserts by digestion with *EcoRI*. Plasmids containing
20 inserts of the correct size were digested with *NheI* and *HindIII* according to the manufacturer's directions and the DNA fragments containing the CT operons isolated from low melting point agarose. Plasmid pBAD18-Cm (Invitrogen) was digested with *NheI-HindIII* and the linear DNA isolated from low melting point agarose. Digested pBAD18 and the CT operons were ligated at 12°C and transformed into Top10F *E. coli*. Plasmids from chloramphenicol-resistant colonies were screened for inserts by
25 restriction analysis, and representative clones were sequenced to confirm the presence of the site directed mutations. Plasmids were transformed into DH5 α for expression of CT-CRMs. The plasmids encoding the mutant CT-CRMs bearing a single amino acid substitution at positions 16 and 72 are designated as pLP903 and pLP905 respectively,
30 and the plasmids encoding the mutant CT-CRMs bearing double amino acid substitution at positions 16 and 68 and at positions 68 and 72 are designated as

pLP904 and pLP906 respectively. The plasmids contain the polycistron of *V. cholerae* genes *ctxA* and *ctxB* which encode the CT.

D. Expression of CT-CRMs in E. coli.

E. coli DH5 α cells containing plasmids pLP903, pLP904, pLP905, or pLP906, 5 cells expressing CT-CRMs CT-CRM_{I16A}, CT-CRM_{S68Y,I16A}, CT-CRM_{V72Y}, and CT-CRM_{S68Y,V72Y} respectively, were grown in phosphate buffered Hy-Soy media containing chloramphenicol (25 μ g/ml) and glycerol (0.5%) at 37°C with aeration. When cultures reached an OD₆₀₀ of approximately 4.5-5.5, they were induced by addition of L-arabinose to a final concentration of 0.5%. Cultures were incubated at 10 37°C with aeration for three hours post-induction and then the cells collected by centrifugation. Cell pellets were stored at -20°C.

E. Preparation and purification of CT-CRMs.

Cell pellets were thawed at room temperature and resuspended in 10 mM NaPO₄ and 1 mM EDTA (pH 7.0) at 9% of the original culture volume. Cell 15 suspensions were mechanically disrupted in a microfluidizer and centrifuged for 10 minutes at 8,500 xg. Cell lysates were further clarified at 160,000 xg for one hour. The clarified cell lysate was loaded, at a flow rate of 2 ml/min, onto a carboxymethyl (CM)-sepharoseTM column (300 ml CM-sepharoseTM per 10 l of culture) (Amersham, Pharmacia) equilibrated with 10 mM NaPO₄ (pH 7.0). The column was washed with 20 >10 volumes of 10 mM NaPO₄ (pH 7.0) at a flow rate of 5 ml/min. CT-CRM_{E29H} holotoxin was eluted with four column volumes of 10 mM NaPO₄ (pH 8.3). Purified CT-CRMs were buffer exchanged by dialysis into PBS and stored at 4°C. The presence of intact holotoxin and the respective subunits was determined by native polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE, respectively. Native 25 PAGE indicated the presence of a purified molecule of 86 kDa (data not shown), the expected molecular weight for intact cholera holotoxin (Tebbey *et al.*, 2000 *Vaccine*, 18 (24): 2723-2734).

In addition, SDS-PAGE showed two bands that aligned with the CT-A (27 kDa) and CT-B (12 kDa) subunits that comprise the intact holotoxin (data not shown).

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EXAMPLE 2: NON-DENATURING POLYACRYLAMIDE GEL
ELECTROPHORESIS

Mutant CT-CRMs, CT-CRM_{I16A,S68Y} and CT-CRM_{V72Y}, were analyzed by non-denaturing page electrophoresis to determine the percentage of the CT-CRMs present after purification as intact holotoxin. Purified CT-CRMs, 15 µl each (at various protein concentrations), were run through a 6% polymerized non-denaturing polyacrylamide gel. Three different concentrations (300, 600 and 1200 ng) of CT-B were used as a standard. After electrophoresis the gel was stained with Coomassie blue. The gel was then scanned using a densitometer, and the percentage of the holotoxin was calculated from the densitometer readings of the CT-CRMs and CT-B standard. The data indicated that 97.8% of CT-CRM_{I16A,S68Y} and 99.4% of CT-CRM_{V72Y} were present as intact holotoxins (Table 3).

Table 3: Native Gel Assay for Intact Holotoxin

CT-CRM	% of holotoxin
CT-CRM _{I16A}	97.8
CT-CRM _{V72Y}	99.4
CT-CRM _{I16A,S68Y}	Not done
CT-CRM _{S68Y,V72Y}	Not done

15 **EXAMPLE 3: Y-1 ADRENAL CELL ASSAY FOR RESIDUAL TOXICITY OF**
CT-CRMS

Mutant CT-CRMs were compared with wild-type CT for toxicity in the mouse Y-1 adrenal tumor cell assay, which is used *in vitro* to measure toxicity of enterotoxins in the cholera toxin/heat labile enterotoxin family. The assay depends upon binding of the toxin to cell surface receptors, and the subsequent entry of the A1 subunit of the toxin into the cytoplasm of the cell.

Native cholera toxin isolated from *V. cholerae* is proteolytically nicked at the CT-A1- CT-A2 junction, resulting in the A1 and A2 subunits of cholera toxin being held together by only a disulfide bond. This makes the A1 and A2 subunits unstable and easily dissociable from each other. The A1 subunit of the nicked CT dissociates from the A2 subunit upon binding cell surface receptor, and enters the cell, where it

ADP-ribosylates the regulatory G-protein (G_{α}), leading to its toxic effects as described in the background above. In contrast, enterotoxins produced in *E. coli* (either CT or LT) are unnicked, and thus, have the A1-A2 peptides still joined. Consequently, the CT produced in *V. cholerae* are significantly more toxic in the Y-1 adrenal cell assays than the CT produced in a heterologous bacterial cell such as *E. coli*.

In a first Y-1 adrenal cell assay, mutant CT-CRMs were compared to nicked wild-type CT from *V. cholerae* for toxicity. In this assay, Y-1 adrenal cells (ATCC CCL-79) were seeded in 96-well flat-bottom plates at a concentration of 10^4 cells per well. Thereafter, three-fold serial dilutions of purified (~90% purity as determined by Coomassie staining) CT-CRMs were added to the tumor cells and incubated at 37°C (5% CO₂) for 18 hours. The cells were then examined by light microscopy for evidence of toxicity (cell rounding). The endpoint titer was defined as the minimum concentration of toxin required for greater than 50% cell rounding. The percent of residual toxicity was then calculated using the endpoint titer of wild-type nicked CT from *V. cholerae* (100% toxicity) divided by the titer elicited by CT-CRMs multiplied by 100. The data set forth in Table 4 indicate that the residual toxicity of the four purified mutant holotoxins, CT-CRM_{I16A}, CT-CRM_{I16A,S68Y}, CT-CRM_{v72Y}, and CT-CRM_{S68Y,V72Y} tested using the Y-1 adrenal cell assay was only 0.37%.

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Table 4 : Y-1 Adrenal Cell Assay

CT-CRM	% Residual Toxicity
CT-CRM _{I16A}	0.37
CT-CRM _{I16A,S68Y}	0.37
CT-CRM _{v72Y}	0.37
CT-CRM _{S68Y,V72Y}	0.37

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In a second independent study, crude periplasmic extracts of *E. coli* cells (TG1) expressing elevated levels of mutant CT-CRMs, were compared against unnicked wild-type CT holotoxin expressed in *E. coli* for residual toxicity in Y-1 adrenal cell assay. Y-1 adrenal cells were incubated in multi-well dishes in an RPMI medium containing 10% fetal calf serum in the presence of crude *E. coli* cell lysate.

Cell toxicity was monitored as before. In this study, one toxic unit was defined as the smallest amount of toxin or supernatant that caused rounding of 75-100% of the cells in a well after overnight incubation. The results of this study are presented in Table 5 below.

5

Table 5: Y-1 Adrenal Cell Assay

CT-CRM	% Residual Toxicity
CT-CRM _{I16A}	5
CT-CRM _{V72Y}	100
CT-CRM _{S68Y,V72Y}	5
CT-CRM _{I16A,S68Y}	Not Determined

The results of this study indicated that while the toxicities of CT-CRM_{I16A} and CT-CRM_{S68Y,V72Y} were substantially reduced (5%), the CT-CRM_{V72Y} was as toxic as the wild-type CT. Without being bound by theory, the variant results in second study (Table 5) may be attributable to the fact that periplasmic crude *E. coli* cell lysates used in the second study contained unnickled mutant CT-CRMs, and to the fact that the toxicity was measured as a percentage of the toxicity of wild-type, unnickled CT produced by *E. coli*. In contrast, the unnickled wild-type CT from *E. coli* has a 50% cell rounding dose of 6250 pg/ml in the Y1 cell assay (data not shown). In the first study, the residual cytotoxicity of the mutant CT-CRMs is expressed as a percentage of the toxicity of wild-type, nicked CT produced by *V. cholerae*, wherein the nicked holotoxin has a 50% cell rounding dose of 125 pg/ml in the Y1 cell assay. Consequently, the residual toxicity reported in the second study is 50 fold higher than that obtained in the first study.

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EXAMPLE 4: THE ADP-RIBOSYLTRANSFERASE ASSAY

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NAD⁺:agmatine ADP-ribosyltransferase activity was measured as the release of [carbonyl-¹⁴C] nicotinamide from radiolabeled NAD⁺. Briefly, CT and CT-CRMs were trypsin activated and incubated for 30 minutes at 30°C with 50 mM glycine/20 mM dithiothreitol in TEAN buffer (Tris/EDTA/sodium azide/sodium chloride) (pH 8.0). Thereafter, the following materials were added to the reaction: 0.1 µg of soybean

trypsin inhibitor, 50 mM potassium phosphate, 10 mM agmatine, 20 mM dithiothreitol, 10 mM magnesium chloride, 100 µM GTP, 3 mM dimyristoylphosphatidyl-choline, 0.2% cholate, 0.03 mg of ovalbumin, 100 µM [adenine-U-¹⁴C]NAD (DuPont NEN™, Boston, MA) and water to a final volume of 300 µl. After incubation for 90 minutes at 5 30° C, 100 µl samples were applied to columns (0.64 x 5 cm) of AG1-X2 (Bio-Rad) that were washed five times with 1.0 ml of distilled/deionized H₂O. Eluates containing [¹⁴C]ADP-ribosylagmatine were collected for radioassay. Mean recovery of ¹⁴C in the eluate is expressed as percentage of that applied to column. The results are presented in Table 6.

10

Table 6: NAD:Agmatine ADP-Ribosyltransferase Activity

CT/CT-CRM	ADP-ribosylagmatine formed (nmol/hr/ µg protein)	% ADP-ribosylation activity
CT, 10 µg	52.5	100
CT-CRM _{I16A}	3.3	2.4
CT-CRM _{I16A,S68Y}	3.4	3.3
CT-CRM _{V72Y}	2.7	1.1
CR-CRM _{S68Y,V72Y}	2.9	1.2

15 ADP-ribosyltransferase activity was also independently determined using diethylamino (benzylidine-amino) guanidine (DEABAG) as a substrate. In this assay, 25 µl aliquots of mutant CT-CRMs from purified cell lysates, activated for 30 minutes at 30°C with 1/50 w/w trypsin, were incubated with 200 µl 2mM DEABAG in 0.1M K₂PO₄, pH 7.5, 10µM NAD, 4 mM DTT for two hours. The reaction was stopped by adding 800 µl of a slurry buffer containing 400 mg DOWEX AG50-X8 resin, to bind 20 unreacted substrate. ADP-ribosylated DEABAG in the supernatant was quantitated by fluorescence emission in a DyNA Quant fluorimeter calibrated with DEABAG. With the exception of the mutant CT-CRM_{V72Y}, ADP ribosyl- transferase activities of the mutant CT-CRMs were substantially reduced over that of wild-type (Table 7). The high level of ADP-ribosyl-transferase activity seen with CT-CRM_{V72Y} may be 25 attributable to the fact that in this study the ADP ribosyl-transferase activity of mutant CT-CRMs was measured using a different substrate in a different assay protocol.

Table 7: ADP-ribosyltransferase Activity of CT-CRMs using Diethylamino (benzylidine-amino) Guanidine (DEABAG)

CT/CT-CRM	% ADP-ribosylation Activity
CT	100
CT-CRM _{S68Y}	11
CT-CRM _{V72Y}	68
CT-CRM _{S68Y,V72Y}	3
CT-CRM _{I16A,S68Y}	Not done

5 **EXAMPLE 5: IMMUNE RESPONSES OF BALB/C MICE IMMUNIZED WITH RECOMBINANT P4 OUTER MEMBRANE PROTEIN (RP4) OF NONTYPABLE HAEMOPHILUS INFLUENZAE (NTHI) ALONE OR IN CONJUNCTION WITH CT-CRMS**

10 In a first experiment, the ability of the mutant CT-CRM_{I16A} to enhance the induction of systemic and mucosal antibodies to recombinant P4 outer membrane protein, (rP4) were assessed. Serum and mucosal anti-P4 antibody titers induced by mutant CT-CRM_{I16A}, were assessed and compared with that of wild-type CT and mutant, CT-CRM_{E29H} (WO 00/18434). In this study, Balb/c mice were immunized intranasally (IN) at weeks 0, 3 and 5 and at week 5, day 6 with a formulation containing 1 µg of recombinant P4 protein in saline or 1 µg of P4 together with 1 µg of wild-type CT, 1 µg of CT-CRM_{E29H} or 0.1, 1, or 10 µg of CT-CRM_{I16A}.

15 The results indicate that the CT-CRM_{I16A}, like the wild-type CT and CT-

CRM_{E29H}, augmented the capacity of rP4 protein to elicit systemic and mucosal immune responses (Table 8). For example, six weeks after primary IN immunization the anti-rP4 IgG antibody titers of mice immunized with rP4 protein formulated with either CT-CRM_{I16A} or CT-CRM_{E29H} were 40 times greater than that of mice immunized with the recombinant proteins in PBS alone. The antibody titers (IgG) of mice administered the recombinant proteins plus wild-type CT holotoxin at a concentration of 1 µg were elevated 67-fold in comparison to antibody titers in mice administered recombinant rP4 alone in saline. The antibody titers of mice immunized with 1 µg of the mutant CT-CRM, CT-CRM_{E29H} were elevated 55-fold over antibody titers in mice

immunized with rP4 alone. In comparison, the antibody titers of mice immunized with 1 µg and 0.1 µg of the mutant CT-CRM, CT-CRM_{II6A}, were increased 15-fold and 27-fold respectively over the anti-rP4 antibody titers in mice immunized with rP4 alone in saline.

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Table 8: Serum Antibody Responses to Recombinant P4 Protein

Adjuvant	Amount	Week 0		Week 3		Week 5		Week 5, Day 6	
		IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
Saline				148	386	199	394	140	444
CT	1.0 µg			184	1,348	203	1,949	1,570	29,939
CT-CRM _{E29H}	1.0 µg			108	615	286	1,057	1,492	21,251
CT-CRM _{II6A}	1.0 µg			<100	426	114	2,071	1,096	6,708
CT-CRM _{II6A}	0.1 µg			261	1,463	403	2,629	1,105	10,541
CT-CRM _{II6A}	10.0 µg	151	502	121	788	108	1,460	183	2,043

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An examination of the protein-specific antibodies in the mucosal secretions of the Balb/c mice immunized IN at weeks 0, 3, and 5 was made two weeks after tertiary immunization. Mucosal samples were collected at week 5, day 6 from vaginal wash (VW), the nasal wash (NW); the bronchoalveolar lavage (BAL) and from saliva (SAL). These results shown in Table 9 further indicated the CT-CRM_{II6A} facilitated the generation of local immune responses against the rP4 protein. Moreover, the anti-rP4 antibody titers were comparable to those induced by the wild-type CT adjuvanted immunogenic composition.

Table 9: Mucosal Antibody Responses to rP4 Protein.

Adjuvant	Amt	VW		NW		BAL		SAL	
		IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
Saline		24	<10	<10	<10	<10	<10	20	<10
CT	1.0 µg	125	74	38	<10	158	152	152	14
CT-CRM _{E29H}	1.0 µg	254	12	58	<10	523	364	454	27
CT-CRM _{II6A}	1.0 µg	154	16	39	<10	330	38	654	32
CT-CRM _{II6A}	0.1 µg	422	26	60	<10	125	53	1027	74
CT-CRM _{II6A}	10.0 µg	19	<10	<10	<10	13	16	34	<10

In a second experiment, five BALB/c mice per group were immunized IN on days 0, 21 and 35 with a 15 µl dose containing 1 µg rP4 alone or 1 µg rP4 plus 1 µg of one of the mutant CT-CRMs, CT-CRM_{E29H}, CT-CRM_{I16A}, CT-CRM_{I16A,S68Y}, CT-CRM_{V72Y} or CT-CRM_{S68Y,V72Y} as an adjuvant as indicated in Table 10. The anti-rP4 IgA and IgG antibody titers were determined by ELISA on pooled samples collected at weeks 0, 3, 5 and week 5, day 6 and the results shown in Table 10. The results indicate that serum anti-rP4, IgA and IgG titers were substantially increased in mice that were administered the antigen along with one of the mutant CT-CRMS. The mucosal antibody responses to rP4 were also measured one week after the last immunization (week 5, day 6).

Table 10: Adjuvant Effects of Mutant Cholera Toxins on the Immune Response to NTHi rP4 Protein Delivered Intranasally to Female BALB/c Mice^a

Adjuvant ^b	Anti-NTHi rP4 ELISA Endpoint Titers on Pooled Sera ^c							
	Week 0		Week 3		Week 5		Week 5, day 6	
	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
Saline	123	593	<100	715	227	1,953	224	6,458
CT-CRM _{E29H}			116	934	1,808	135,824	7,365	282,099
CT-CRM _{I16A}			269	423	788	17,465	2,609	143,313
CT-CRM _{I16A,S68Y}			256	545	821	6,062	1,998	438,553
CT-CRM _{V72Y}			294	878	1,725	40,443	5,239	343,711
CT-CRM _{S68Y,V72Y}			172	429	333	4,353	1,333	55,571

^a 1µg NTHi rP4 was delivered IN to female BALB/c mice in a 15 µl volume at weeks 0, 3 and 5.

^b NTHi rP4 compositions were formulated with saline or 1 µg of various mutant cholera toxins.

^c Sera were collected at weeks 0, 3, 5 and week 5, day 6; pooled samples represent an n=5.

Table 11 sets forth the IgA and IgG titers from nasal, bronchoalveolar and vaginal washes, and saliva respectively. These results also indicate that mucosal anti-rP4, IgA and IgG titers were also substantially elevated in mice administered rP4 antigen together with one of the mutant CT-CRMs in comparison to mice administered rP4 in saline.

Table 11: Anti-NTHi rP4 ELISA Endpoint Titers on Mucosal Wash Pools^a

Adjuvant ^b	Lung Wash ^c		Nasal Wash ^c		Saliva ^c		Vaginal Wash ^c	
	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
Saline	<10	65	<10	<10	12	<10	<10	<10
CT-CRM _{E29H}	1,131	1,815	196	70	977	1,906	255	149
CT-CRM _{I16A}	360	708	166	37	733	96	1,066	414
CT-CRM _{I16A,s68Y}	449	376	164	19	1,221	177	1,521	261
CT-CRM _{V72Y}	3,850	4,226	500	300	2,753	281	1,066	212
CT-CRM _{s68Y,V72Y}	84	164	111	35	843	103	251	109

^a 1 µg NTHi rP4 was delivered IN to female BALB/c mice in a 15 µl volume at weeks 0, 3 and 5.

^b NTHi rP4 compositions were formulated with saline or 1 µg of various mutant cholera toxins.

^c Mucosal samples were collected on week 6; pooled samples represent an n = 5.

On week 5, day 6, following IN administration, IgA and IgG including IgG subclass IgG1, IgG2a, IgG2b and IgG3 endpoint titers in the serum of each individual mouse in the six groups were also determined by ELISA. (Tables 12-17). In the data reported in Tables 12-17, statistical analyses were performed using JMP, SAS Institute, Inc.; one-way analysis of variance was significant at the p<.0001 level; and multiple comparisons were performed using Tukey-Kramer HSD, alpha = 0.05.

Table 12: IgA Anti-NTHi rP4 ELISA Endpoint Titers in each Individual Mouse at Week 5, Day 6.

Adjuvant	1	2	3	4	5	GMT	StDev	SEM
Saline	217	63	44	48	35	63	76	34
CT-CRM _{E29H}	6,791	2,196	1,740	1,526	4,626	2,835	2,276	1,018
CT-CRM _{I16A}	2,075	2,186	1,208	2,847	1,975	1,985	585	262
CT-CRM _{I16A,s68Y}	1,739	920	739	1,289	5,694	1,541	2,058	921
CT-CRM _{V72Y}	6,523	1,634	1,290	4,089	10,521	3,584 [†]	3,826	1,711
CT-CRM _{s68Y,V72Y}	1,646	878	310	2,724	116	676*	1,069	478

* Values differ significantly from the saline group

[†] Value differs significantly from the CT-CRM_{s68Y,V72Y} group.

Table 13: IgG Anti-NTHi rP4 ELISA Endpoint Titers in Each Individual Mouse at Week 5, Day 6.

Adjuvant	1	2	3	4	5	GMT	StDev	SEM
Saline	78,369	2,287	3,505	1,518	1,223	4,105*	34,105	15,252
CT-CRM _{E29H}	440,101	280,030	17,291	111,803	193,831	135,797*	161,927	72,416
CT-CRM _{I16A}	69,986	92,347	68,406	193,467	62,196	88,141*	54,968	24,582
CT-CRM _{I16A,s68Y}	57,721	59,832	17,521	57,172	29,725	40,034*	19,471	8,708
CT-CRM _{V72Y}	161,796	143,545	72,764	187,118	363,958	163,008*	108,260	48,415
CT-CRM _{s68Y,V72Y}	80,770	71,053	14,366	73,904	2,658	27,662*	36,953	16,526

* Values differ significantly from the saline group

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Table 14: IgG1 Anti-NTHi rP4 ELISA Endpoint Titers in Each Individual Mouse on Week 5, Day 6 Sera.

Adjuvant	1	2	3	4	5	GMT	StDev	SEM
Saline	2,533	33	33	33	33	79	1,118	500
CT-CRM _{E29H}	11,462	3,029	1,801	8,517	1,172	3,623*	4,547	2034
CT-CRM _{I16A}	4,732	10,033	3,546	14,399	6,836	6,980*	4,385	1961
CT-CRM _{I16A,s68Y}	2,945	1,036	1,016	2,945	343	1,256*	1,208	540
CT-CRM _{V72Y}	9,123	13,942	545	7,939	35,887	7,230*	13,410	5997
CT-CRM _{s68Y,V72Y}	28,434	13,553	394	7,579	33	2,070*	11,725	5244

* Values differ significantly from the saline group

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Table 15: IgG2a Anti-NTHi rP4 ELISA Endpoint Titers in Each Individual Mouse Week 5, Day 6

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Adjuvant	1	2	3	4	5	GMT	StDev	SEM
Saline	10,611	317	431	230	217	591	4,613	2063
CT-CRM _{E29H}	230,644	189,940	3,489	43,336	132,159	61,439*	95,830	42858
CT-CRM _{I16A}	24,886	43,309	42,582	89,228	23,210	39,408*	26,663	11924
CT-CRM _{I16A,s68Y}	39,555	37,700	6,947	29,050	17,604	22,122*	13,814	6178
CT-CRM _{V72Y}	125,810	75,952	41,286	108,108	210,157	97,834*	63,522	28409
CT-CRM _{s68Y,V72Y}	10,136	27,702	6,289	30,058	1,462	9,505*	12,950	5792

* Values differ significantly from the saline group

Table 16: IgG2b Anti-NTHi rP4 ELISA Endpoint Titers in Each Individual Mouse on Week 5, Day 6 Sera

Adjuvant	1	2	3	4	5	GMT	StDev	SEM
Saline	5,473	132	153	102	66	237	2,397	1072
CT-CRM _{E29H}	85,041	70,413	529	8,597	36,760	15,852	37,108	16596
CT-CRM _{IL6A}	10,554	15,798	7,562	27,484	7,885	12,227*	8,302	3713
CT-CRM _{IL6A,s68Y}	13,545	9,942	2,731	8,724	6,257	7,253*	4,048	1810
CT-CRM _{V72Y}	25,782	23,712	14,433	25,347	74,216	27,798*	23,664	10583
CT-CRM _{s68Y,V72Y}	5,096	14,958	3,205	16,081	582	4,697*	7,074	3164

* Values differ significantly from the saline group

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Table 17: IgG3 Anti-NTHi rP4 ELISA Endpoint Titers in Each Individual Mouse on Week 5, Day 6 Sera

Adjuvant	1	2	3	4	5	GMT	StDev	SEM
Saline	992	33	33	33	33	65	429	192
CT-CRM _{E29H}	311	36	23	344	1,246	162	500	224
CT-CRM _{IL6A}	2,256	62	390	290	150	299	918	411
CT-CRM _{IL6A,s68Y}	120	1,953	33	612	64	198	816	365
CT-CRM _{V72Y}	479	929	25	1,124	462	357	432	193
CT-CRM _{s68Y,V72Y}	202	69	3	92	33	42	76	34

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EXAMPLE 6: THE IMMUNE RESPONSES OF BALB/C MICE IMMUNIZED WITH THE PURIFIED NATIVE FUSION (F) GLYCOPROTEIN OF RESPIRATORY SYNCYTIAL VIRUS (RSV)

The capacity of the CT-CRMs of the present invention to augment systemic and mucosal immune responses against respiratory syncytial virus (RSV) glycoproteins was examined using the purified native fusion (F) protein. Previously it was demonstrated that BALB/c mice immunized IN with F protein adjuvanted with either CT or CT-CRM_{E29H} generated systemic and local IgG and IgA titers (Tebbey *et al*, cited above). That study also indicated that pre-existing anti-CT antibodies did not have a negative impact on the level of local or systemic anti-F protein IgA and IgG antibodies. Indeed, the study indicated that pre-existing anti-CT antibodies were beneficial for the generation of an augmented anti-F protein antibody response. Additionally, the data also suggested a mechanism involving the neutralization of

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infectious virus by either mucosal or humoral immunoglobulins that were stimulated in response to the IN immunization protocol containing F/CT-CRM_{E29H}.

BALB/c mice (5 per group) were immunized (IN, 0.01 ml) at weeks 0 and 3 with native purified F protein (3 µg/dose) alone in saline or in a formulation containing 5 0.1 or 1 µg of one of the wild-type CT (Sigma) or 0.1 or 1 µg of one of the genetically detoxified mutant CT-CRMs (CT-CRM_{E29H}, CT-CRM_{I16A}, CT-CRM_{I16A,S68Y}, CT-CRM_{V72Y} and CT-CRM_{S68Y,V72Y}). Sera were collected 2 weeks post secondary immunization. The titration of the protein-specific IgG, IgA and IgG subclass, serum neutralizing, antibodies in the bronchoalveolar wash, nasal wash and vaginal wash was 10 performed in duplicate on HEp-2 cell monolayers in 96-well microplates. Furthermore, a subgroup of immunized mice was challenged with live virus to determine the protective capacity of the immunogenic formulations. The results are presented in Table 20. The numbers are geometric mean endpoint anti-F protein IgG, subclass and IgA antibody titers (\pm 1 standard deviation).

15 Analysis of serum antibodies post-secondary immunization showed that immunization with any of the cholera toxin-derived adjuvants significantly induced immune responses to RSV F protein (Table 18). The use of each of the cholera toxin mutants CT-CRM_{I16A}, CT-CRM_{I16A,S68Y}, CT-CRM_{V72Y}, CT-CRM_{S68Y,V72Y} and CT-CRM_{E29H}, at concentrations of 0.1 or 1.0 µg/dose significantly ($p < 0.05$) induced serum 20 antibodies (total IgG, IgG1, IgG2a and IgA) to RSV F protein. The magnitude of the total IgG immune response to RSV F protein was increased approximately 25-fold by inclusion of the cholera toxin-derived adjuvants when compared to the response achieved by animals administered a composition containing F/PBS.

25 Statistical significance of the data reported in Table 18 below is as follows. For total IgG: $p < 0.05$: F/PBS vs.. All. $p > 0.05$: F/CT vs.. F/CT-CRM_{E29H} vs.. F/CT-CRM_{I16A} vs. F/CT-CRM_{I16A,S68Y} vs. F/CT-CRM_{S68Y,V72Y} vs. FCT-CRM_{S68Y,V72Y} (at both 0.1 and 1.0 µg/dose). For IgG1: $p < 0.05$: F/PBS vs. All. $p > 0.05$: F/CT vs. F/CT-CRM_{E29H} vs. F/CT-CRM_{I16A} vs. F/CT-CRM_{I16A,S68Y} vs. F/CT-CRM_{V72Y} vs. F/CT-CRM_{S68Y,V72Y} (at both 0.1 and 1.0 µg/dose). For IgG2a: $p < 0.05$: F/PBS vs. All. F/CT (0.1 µg) vs. F/CT-CRM_{I16A} (0.1 µg). $p > 0.05$: At 1.0 µg dose, F/CT vs. F/CT-CRM_{E29H} vs. F/CT-CRM_{I16A} vs. F/CT-CRM_{I16A,S68Y} vs. F/CT-CRM_{V72Y} vs. F/CT-

CRM_{S68Y,V72Y}. At 0.1 µg dose, F/CT vs. F/CT-CRM_{E29H} vs. F/CT-CRM_{IL6A,S68Y} vs. F/CT-CRM_{V72Y} vs. F/CT-CRM_{S68Y,V72Y}.

No significant differences were observed in total anti-F IgG or IgG1 titers between each of the new mutant toxins (CT-CRM_{IL6A}, CT-CRM_{IL6A,S68Y}, CT-CRM_{V72Y}, and CT-CRM_{S68Y,V72Y}) and either CT_{E29H} or wild-type CT. Evidence of statistical differences was observed between specific groups upon analysis of IgG2a and IgA titers. However, these comparisons did not reveal any consistent trends regarding the immunological performance of one mutant versus another.

**Table 18: The Humoral Immune Responses of BALB/c Mice after
Intranasal Immunization with F Protein and Mutant CT-CRMs**

Antigen	Adjuvant (µg)	IgG	IgG1	IgG2a	IgA
F protein	None	4.1 ± 0.6	3.1 ± 0.4	1.9 ± 0.3	1.9 ± 0.4
F protein	CT-CRM _{E29H} (1)	6.3 ± 0.7	5.8 ± 0.8	4.9 ± 0.7	4.3 ± 0.6
F protein	CT-CRM _{E29H} (0.1)	5.6 ± 0.2	5.4 ± 0.5	4.4 ± 0.2	4.0 ± 0.2
F protein	CT-CRM _{IL6A} (1)	5.6 ± 1.6	5.2 ± 1.4	4.7 ± 1.1	4.3 ± 0.2
F protein	CT-CRM _{IL6A} (0.1)	5.5 ± 0.3	5.0 ± 0.1	3.9 ± 0.4	3.5 ± 0.5
F protein	CT-CRM _{V72Y} (1)	6.4 ± 0.2	5.8 ± 0.3	5.3 ± 0.3	4.5 ± 0.3
F protein	CT-CRM _{V72Y} (0.1)	5.6 ± 0.2	5.2 ± 0.4	4.1 ± 0.4	3.5 ± 0.2
F protein	CT-CRM _{IL6A,V72Y} (1)	6.4 ± 0.1	5.7 ± 0.2	5.3 ± 0.3	4.6 ± 0.4
F protein	CT-CRM _{IL6A,V72Y} (0.1)	5.5 ± 0.04	5.5 ± 0.3	4.8 ± 0.2	4.1 ± 0.3
F protein	CT-CRM _{S68Y,V72Y} (1)	6.5 ± 0.1	5.6 ± 0.2	4.9 ± 0.3	4.8 ± 0.2
F protein	CT-CRM _{S684,V72Y} (0.1)	5.8 ± 0.3	5.6 ± 0.2	4.2 ± 0.3	4.6 ± 0.2
F Protein	CT(1)	6.7 ± 0.3	6.0 ± 0.3	5.7 ± 0.2	5.3 ± 0.5
F protein	CT(0.1)	5.8 ± 0.1	5.7 ± 0.2	5.0 ± 0.4	4.9 ± 0.2

In another experiment, groups of 5 BALB/c mice were immunized (IN, 0.01 ml) at weeks 0 and 3 with native F protein (3 µg/dose). The F protein was admixed with 1 or 0.1 µg of genetically detoxified mutants or wild-type CT. Anti-F protein antibody responses were also analyzed in pooled mucosal wash samples of
5 bronchoalveolar lavage (BAL), nasal wash (NW) and vaginal wash (VW), collected 2 weeks post-secondary immunization (Table 19). The data represent endpoint anti-F protein IgG and IgA antibody titers of pooled samples. As expected, no induction of antibody in mucosal washes from F/PBS immunized mice was observed. However, the potent mucosal adjuvant capacity of each mutant cholera holotoxin was readily apparent. Although no statistical analyses were performed on these pooled samples, some trends surfaced. For example, mice that received F/CT-CRM_{V72Y} (1.0 µg) displayed elevated IgG and IgA in each of the BAL, NW and VW samples taken. In comparison, mutant toxins CT-CRM_{I16A}, CT-CRM_{I16A,S68Y} and CT-CRM_{S68Y,V72Y} appeared to be comparable to CT-CRM_{E29H} in adjuvanting local immune responses to
10 RSV F protein.
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Table 19: The Mucosal Immune Responses of BALB/c Mice after Intranasal Immunization with F Protein and Genetically Detoxified Mutants

Antigen	Adjuvant (μ g)	BAL		NW		VW	
		IgG	IgA	IgG	IgA	IgG	IgA
F protein	None	<25	<25	<25	<25	<25	<25
F protein	CT-CRM _{E29H} (1)	1569	211	320	793	265	1629
F protein	CT-CRM _{E29H} (0.1)	549	<25	45	136	99	202
F protein	CT-CRM _{II6A} (1)	1349	43	415	287	325	1427
F protein	CT-CRM _{II6A} (0.1)	376	<25	103	187	217	350
F protein	CT-CRM _{V72Y} (1)	1177	121	314	280	222	2289
F protein	CT-CRM _{V72Y} (0.1)	144	<25	51	71	48	311
F protein	CT-CRM _{II6A,V72Y} (1)	2093	458	392	627	739	9683
F protein	CT-CRM _{II6A,V72Y} (0.1)	499	39	133	785	134	500
F protein	CT-CRM _{S68Y,V72Y} (1)	1248	79	1181	510	204	1374
F protein	CT-CRM _{S68Y,V72Y} (0.1)	522	25	109	98	81	770
F protein	CT(1)	3271	142	1593	710	619	4136
F protein	CT(0.1)	6436	1037	395	362	1185	1100

5 In another experiment, BALB/c mice were immunized (IN, 0.01 μ l) at weeks 0 and 3 with native F protein (3 μ g/dose). The F protein was admixed with 1 or 0.1 μ g of each genetically detoxified mutant or wild-type CT. At week 5, mice were challenged with the A2 strain of RSV and lungs harvested 4 days later to quantitate virus infectivity. Each of the mutant cholera holotoxins induced a protective immune response to RSV challenge as measured by viral lung load (Table 20). Data are presented as the mean virus recovered (\log_{10})/g tissue. Neutralizing antibodies were assayed in the presence of 5% guinea pig serum as a source of complement (C') in bleeds taken two weeks post-secondary immunization. Data show the mean titer (\log_{10}) which showed a 60% reduction in pfu/well compared to control wells.

10 The statistical analyses of the data from the virus infectivity assays is reported as $p < 0.05$: F/PBS vs. all; $p > 0.05$: F/CT-E29H vs. F/CT vs. F/CRM/_{II6A} vs. F/CT-CRM_{V72Y} vs. F/CT-CRM_{II6A,V72Y} vs. F/CT-CRM_{S68Y,V72Y} at both 0.1 and 1.0 μ g/dose.

Serum neutralizing responses: p < 0.05: F/PBS vs. all except F/CT-CRM_{II6A,S68Y} (0.1 µg). F/CT(1.0) vs. F/CT-CRM_{II6A} (0.1). F/CT-CRM_{II6A,S68Y} (0.1) vs. F/CT-CRM_{II6A,S68Y} (1.0) F/CT (9.0), F/CT-CRM_{II6A} (1.0), F/CT-CRM_{E29H} (1.0), F/CT-CRM_{V72Y} (0.1).

5 Lungs from mice immunized with F/PBS were clearly populated with RSV ($\log_{10}3.4$ pfu/g tissue). In contrast, those mice immunized with F protein co-formulated with mutant cholera holotoxins displayed no detectable virus. A somewhat similar pattern was observed for serum neutralizing antibodies (Table 20). Those mice immunized with F/PBS displayed complement-assisted neutralizing antibodies that
10 were significantly reduced compared to all mice that had received mutant cholera holotoxins as an adjuvant except F/CT-CRM_{II6A,S68Y} at 0.1 µg per dose. Whereas evidence of neutralizing activity was observed in the absence of complement, no statistical differences were observed (Table 20). Collectively these data suggest that the inclusion of the mutant cholera holotoxins CT-CRM_{II6A}, CT-CRM_{II6A,S68Y}, CT-
15 CRM_{V72Y}, and CT-CRM_{S68Y,V72Y} contributes substantially to the magnitude of the functional immune responses to RSV F protein.

Table 20: Functional Immune Responses Elicited by Immunization with Purified F Protein and Mutant CT-CRMs

Antigen	Adjuvant (μg)	GMT pfu/g (Log ₁₀)	Geometric Mean Serum (Log ₁₀)	
		Lung Tissue	+ C'	- C'
F protein	None	3.38 ± 0.72	1.1 ± 0.2	< 1.3
F protein	CT-CRM _{E29H} (1)	<1.5 ± 0.03	3.0 ± 0.6	1.6 ± 0.6
F protein	CT-CRM _{E29H} (0.1)	<1.5 ± 0.1	2.7 ± 0.5	< 1.3
F protein	CT-CRM _{I16A} (1)	<1.6 ± 0.03	3.1 ± 0.3	1.5 ± 0.7
F protein	CT-CRM _{I16A} (0.1)	<1.5 ± 0.03	2.3 ± 0.7	< 1.3
F protein	CT-CRM _{V72Y} (1)	<1.6 ± 0.05	2.9 ± 0.5	1.6 ± 0.6
F protein	CT-CRM _{V72Y} (0.1)	<1.5 ± 0.03	1.8 ± 0.4	1.1 ± 0.1
F protein	CT-CRM _{I16A,V72Y} (1)	<1.5 ± 0.02	2.7 ± 0.7	1.4 ± 0.6
F protein	CT-CRM _{I16A,V72Y} (0.1)	<1.5 ± 0.05	3.1 ± 0.2	1.5 ± 0.6
F protein	CT-CRM _{S68Y,V72Y} (1)	<1.6 ± 0.05	2.5 ± 0.6	1.4 ± 0.6
F protein	CT-CRM _{S68Y,V72Y} (0.1)	<1.5 ± 0.5	2.5 ± 0.5	< 1.3
F protein	CT(1)	<1.5 ± .07	3.5 ± 0.4	1.4 ± 0.6
F protein	CT(0.1)	<1.6 ± 0.06	2.6 ± 0.5	1.5 ± 0.6

5 In yet additional experiments, naïve BALB/c mice (8-10 weeks of age, 5/group) were immunized (IN, 10 μl) at weeks 0 and 3 with native purified fusion (F) protein purified from the 248/404 strain of RSV. The protein (3 μg/dose) was prepared in mixture with 1.0 or 0.1 μg of the indicated CT-CRM. Control mice were immunized with F protein admixed with CT-CRM_{E29H} alone, with wild-type CT, or with PBS. Serum (geometric mean titer ± 1 standard deviation) and bronchoalveolar (BAW), nasal (NW) and vaginal (VW) wash fluids were collected two weeks after secondary immunization for the determination of end-point anti-F protein total and subclass IgG and IgA titers by ELISA. The mucosal wash samples were pooled for the determination of endpoint titers.

10 15 The results from two experiments are presented in Tables 21 and 22.

Table 21: Geometric Serum Ig Titers of BALB/c Mice Immunized with F Protein Formulated with the Mutant CT-CRMs

Antigen	Adjuvant (µg)	Anti-F Protein Ig Titers (Log_{10})			
		IgG	IgG1	IgG2a	IgA
F protein	None	4.0 ± 0.7	2.7 ± 1.0	2.3 ± 0.7	2.3 ± 0.7
F protein	CT-CRM _{I16A} (1)	5.4 ± 0.3	5.1 ± 0.2	5.1 ± 0.3	4.3 ± 0.3
F protein	CT-CRM _{I16A} (0.1)	5.1 ± 0.4	4.6 ± 0.4	4.0 ± 0.5	3.9 ± 0.5
F protein	CT-CRM _{I16A,S68Y} (1)	5.7 ± 0.2	5.4 ± 0.3	5.3 ± 0.3	4.7 ± 0.3
F protein	CT-CRM _{I16A,S68Y} (0.1)	5.2 ± 0.3	5.5 ± 0.3	4.2 ± 0.2	4.0 ± 0.2
F protein	CT-CRM _{V72Y} (1)	5.6 ± 0.1	5.4 ± 0.2	5.0 ± 0.2	4.7 ± 0.1
F protein	CT-CRM _{V72Y} (0.1)	5.3 ± 0.3	4.8 ± 0.2	4.7 ± 0.2	4.6 ± 0.2
F protein	CT-CRM _{S68Y,V72Y} (1)	5.7 ± 0.2	5.5 ± 0.3	4.3 ± 0.3	4.4 ± 0.2
F protein	CT-CRM _{S68Y,V72Y} (0.1)	5.1 ± 0.2	4.5 ± 0.4	4.0 ± 0.3	4.1 ± 0.3
F protein	CT-CRM _{E29H} (1)	5.4 ± 0.3	5.4 ± 0.1	5.5 ± 0.6	4.6 ± 0.3
F protein	CT-CRM _{E29H} (0.1)	5.3 ± 0.4	5.4 ± 0.1	4.3 ± 0.4	4.3 ± 0.1
F protein	CT(1)	5.4 ± 0.5	5.0 ± 0.5	4.4 ± 0.8	4.6 ± 1.0
F protein	CT(0.1)	4.6 ± 0.3	4.5 ± 0.4	3.5 ± 0.2	4.3 ± 0.3

Table 22. The Ig Titers of Pooled Mucosal Wash Samples from BALB/c Mice Immunized with F Protein Formulated with Mutant CT-CRMs

Antigen	Adjuvant (μg)	Anti-F Protein Ig Titers					
		BAW		NW		VW	
		IgG	IgA	IgG	IgA	IgG	IgA
F protein	None	<25	<25	<25	157	<25	44
F protein	CT-CRM _{I16A} (1)	1,177	60	1,062	1,319	270	1,773
F protein	CT-CRM _{I16A} (0.1)	340	75	280	228	57	8,008
F protein	CT-CRM _{I16A,S68Y} (1)	6,029	917	656	1,543	1,200	7,660
F protein	CT-CRM _{I16A,S68Y} (0.1)	2,318	1,028	273	415	669	5,904
F protein	CT-CRM _{V72Y} (1)	5,879	497	6,327	1,940	1,325	4,632
F protein	CT-CRM _{V72Y} (0.1)	4,696	2,954	764	1,002	1,726	684
F protein	CT-CRM _{S68Y,V72Y} (1)	2,179	364	444	4,153	907	849
F protein	CT-CRM _{S68Y,V72Y} (0.1)	1,030	125	289	646	440	201
F protein	CT-CRM _{E29H} (1)	1,972	217	616	437	327	43,466
F protein	CT-CRM _{E29H} (0.1)	1,893	222	1,993	1,013	845	5,489
F protein	CT (1)	2,189	434	269	474	1,308	994
F protein	CT(0.1)	1,791	308	316	1,997	315	358

When the CT-CRM mutants of this invention were used as mucosal adjuvants at the 1.0 μg dose, results similar to the use of mutant CT-CRM_{E29H} or wild-type CT were obtained (Table 21). Noteworthy differences from the anti-F protein IgG or IgA titers elicited following immunization with F protein admixed with CT-CRM_{E29H} or wild-type CT were not observed. Because pooled samples were used to determine Ig titers in mucosal wash fluids, statistical analyses could not be performed. Nonetheless, the titers elicited by the mutant CT-CRMs of this invention were comparable to those induced by F protein admixed with CT-CRM_{E29H} or wild-type CT (Table 22).

Thus, all CT-CRM mutants of this invention had adjuvant activity for F protein.

EXAMPLE 7: THE IMMUNE RESPONSES OF BALB/C MICE IMMUNIZED WITH THE USPA2 OUTER MEMBRANE PROTEIN OF *M. CATARRHALIS*

In this study, the capacity of mutant CT-CRMs to augment systemic and mucosal immune responses against the native UspA2 outer membrane protein of *M. catarrhalis* was examined. Purified UspA2 (5 µg/dose) alone in 10 µl saline or in a 10 µl formulation containing 0.1 µg/dose of a mutant CT-CRM (CT-CRM_{E29H}, CT-CRM_{I16A}, CT-CRM_{I16A,S68Y}, CT-CRM_{V72Y} or CT-CRM_{S68Y,V72Y}) was administered to Balb/c mice IN on days 0, 7 and 14. Protein-specific IgG and IgA levels in the serum and in mucosal lavages were examined on day 28. The resulting serum and mucosal IgG titers are shown in Table 23. All mutant CT-CRMs, except CT-CRM_{I16A}, elicited enhanced serum IgG antibody response. The levels of IgG and IgA in bronchial, nasal and vaginal washes were measured. No IgA was detected in any of the washes, and IgG was detected only in a few washes.

15 **Table 23: Titers of sera to UspA2 elicited in mice by UspA2 administered intranasally with different CT-CRMs.**

CT Mutants	Serum Antibodies		Mucosal IgG antibodies		
	IgG (\log_{10} titer ISD)	IgA	Lung	Nose	Vagina
None	530 (2.724±0.38)	<10	<10	<10	<10
CT-CRM _{E29H}	17,378 (4.24±0.47)	37	35	<10	<10
CT-CRM _{I16A}	548 (2.739±0.48)	<10	<10	<10	<10
CT-CRM _{I16A,S68Y}	7943 (3.90±1.15)	45	23	<10	15
CT-CRM _{V72Y}	9550 (3.98±0.82)	42	45	<10	19
CT-CRM _{S68Y,V72Y}	1072 (3.03±0.89)	<10	<10	<10	<10

EXAMPLE 8: ADJUVANTICITY OF MUTANT CHOLERA TOXIN HOLOTOXINS

To create a comprehensive panel of mutant CT-CRMs with different characteristics of toxicity, functionality and immunogenicity, the above-described CT-CRM mutants were analyzed as mucosal adjuvants, and the toxicity and enzymatic activity profiles of each of the mutants were determined. As summarized in Table 24,

all of the mutant CT-CRMs have significantly reduced toxicity and enzyme activity compared to wild-type CT. The following data was generated from two studies performed to evaluate these genetically detoxified mutant CTs for their capacity to adjuvant immune responses to native UspA2 protein from *M. catarrhalis*.

5 The experiments were performed as follows: BALB/c mice (6 – 8 weeks old, 5 mice/group) were immunized at weeks 0, 2 and 4 with 5 µg of purified native UspA2 protein in PBS or co-formulated with 1 µg of wild-type CT, or CT-CRM_{E29H}, or CT-CRM_{II6A}, or CT-CRM_{V72Y}, or CT-CRM_{II6A,S68Y}, or CT-CRM_{S68Y,V72Y} per immunization. A total volume of 10 µl was administered intranasally (5 µl per nostril).
10 Mice were bled at weeks 0, 2, 4, or 6 in order to assay serum antibody responses. Two weeks after the last immunization (week 6), mice were sacrificed for the analysis of mucosal antibody responses. Significant differences between groups were determined by the Tukey-Kramer HSD multiple comparisons test using JMP® statistical discovery software (SAS Institute Inc., Cary, NC.).

15 Adjuvanticity of the CT-CRMs can be summarized as follows. Analysis of serum antibodies at week 6 showed that immunization with UspA2 protein formulated with any of the CT-CRM mutants, at a concentration of 1 µg/dose, significantly induced IgG antibody responses to UspA2 protein. The magnitude of the total IgG antibody response to UspA2 protein was increased approximately 17 - 38 fold by inclusion of the CT-derived mutants (excluding CT-CRM_{II6A,S68Y}) (Table 25). No significant differences were observed in total anti-UspA2 IgG titers between the mutant toxins, CT-CRM_{II6A}, CT-CRM_{V72Y}, and CT-CRM_{S68Y,V72Y} and CT-CRM_{E29H}, even though they all elicited significantly higher IgG titers than UspA2 protein alone by Tukey-Kramer HSD test (Table 25). The use of each of the CT-CRM mutants also enhanced serum IgG subclass antibodies (IgG1, IgG2a and IgG2b) to UspA2 protein (Table 27). The ratio of IgG1 and IgG2a or IgG2b titers was approximately 1.0, indicating a balanced Th1/Th2 type of immune response.

20
25
30 Anti-UspA2 protein antibody responses were also analyzed in pooled mucosal wash samples (Table 27). As expected, no induction of antibody in bronchoalveolar lavage (BAL), nasal washes (NW), vaginal washes (VW) or saliva from UspA2/PBS immunized mice was observed. However, the potent mucosal adjuvant capacity of

CT-CRM_{I16A}, CT-CRM_{V72Y}, CT-CRM_{S68Y,V72Y}, and CT-CRM_{I16A,S68Y} was clearly shown. There were UspA2 specific mucosal IgA antibodies detected in most of the mucosal samples. Although no statistical analysis can be performed on these pooled samples, some trends appeared. For example, mice that received CT-CRM_{V72Y}

5 displayed elevated UspA2 specific IgA antibodies in each of the NW, VW and saliva samples tested.

CT-CRM_{I16A}, CT-CRM_{V72Y}, and CT-CRM_{S68Y,V72Y} are potent mucosal adjuvants for *M. catarrhalis* UspA2 protein. The serum antibody data showed that all the CT-CRMs except CT-CRM_{I16A,S68Y} at 1 µg dose are equally as capable in

10 adjuvanting immune responses to UspA2 protein as is CT-CRM_{E29H} (Tables 25 and 26). The mucosal wash data appears to suggest that all the mutant CT-CRMs retain potent mucosal adjuvant properties (Table 27). Furthermore, they all have significantly lower residual toxicity and enzyme activity than wild-type CT, as shown in Table 24. Therefore, CT-CRM_{I16A}, CT-CRM_{V72Y}, CT-CRM_{S68Y,V72Y} and CT-CRM_{I16A,S68Y} are

15 additional effective mucosal adjuvants.

Table 24. Characterization of Mutant Cholera Toxins

Mutant CT	Homogen- eity (%)	Holotoxin (%)	Y-1 cell toxicity (%)	ADP-Ribosyl- transferase activity (%)
CT-CRM _{I16A}	> 90	Not done	0.37	3.3
CT-CRM _{I16A,S68Y}	75.8	97.8	0.37	2.4
CT-CRM _{V72Y}	95.5	99.4	0.37	1.1
CT-CRM _{S68Y,V72Y}	78.9	Not done	0.37	1.2

20 Groups of five female BALB/c mice were immunized intranasally at weeks 0, 2, and 4 with 10µL containing 5µg nUspA2 adjuvanted with 1µg CT (Sigma) or CT mutants. Endpoint antibody titers were determined from sera collected at week 5 day 6. Data are presented as the geometric mean (± 1 SD) of the reciprocal dilution resulting in an OD₄₀₅ of 0.1. Statistical analysis was by Tukey-Kramer. The results are

25 shown in Table 25.

Table 25: The serum anti-nUspA2 responses of BALB/c mice after intranasal immunization with nUspA2 adjuvanted with mutant CTs

Group	Antigen (5µg)	Adjuvant (1 µg)	Mean log 10 Antibody Titers (± 1SD)	
			IgG	IgA
AG414	nUspA2	PBS	<2.00	<2.00
AH415	nUspA2	CT	4.08 ± 0.20*	2.47 ± 0.33
AH416	nUspA2	CT-CRM _{E29H}	3.37 ± 0.37*	2.04 ± 0.09
AH417	nUspA2	CT-CRM _{I16A}	3.23 ± 0.21*	2.00 ± 0.02
AH418	nUspA2	CT-CRM _{I16A,S68Y}	2.63 ± 0.12* [◊]	<2.00
AH419	nUspA2	CT-CRM _{V72Y}	3.59 ± 0.27*	2.11 ± 0.15
AH420	nUspA2	CT-CRM _{S68Y,V72Y}	3.41 ± 0.22*	<2.00

*Significantly higher than the nUspA2/PBS group

5 ◊ Significantly lower than all other adjuvanted groups

Groups of five female BALB/c mice were immunized intranasally at weeks 0, 2, and 4 with 10µL containing 5µg nUspA2 adjuvanted with 1µg CT (Sigma) or CT mutants. Endpoint antibody titers were determined from sera collected at week 5 day 6. Data are presented as the geometric mean (±1 SD) of the reciprocal dilution resulting in an OD₄₀₅ of 0.1. Statistical analysis was by Tukey-Kramer. The results are shown in Table 26.

Table 26. The serum anti-nUspA2 responses of BALB/c mice after intranasal immunization with nUspA2 adjuvanted with mutant CT-CRMs

Group	Antigen (5µg)	Adjuvant (1 µg)	Mean log 10 Antibody Titers (± 1SD)		
			IgG1	IgG2a	IgG2b
AG414	nUspA2	PBS	<2.00	<2.00	<2.00
AH415	nUspA2	CT	3.27 ± 0.12*	3.39 ± 0.34*	3.03 ± 0.17*
AH416	nUspA2	CT-CRM _{E29H}	2.60 ± 0.08*	2.82 ± 0.32*	2.68 ± 0.25*
AH417	nUspA2	CT-CRM _{I16A}	2.30 ± 0.20	2.91 ± 0.24*	2.54 ± 0.21*
AH418	nUspA2	CT-CRM _{I16A,S68Y}	2.36 ± 0.09*	2.65 ± 0.21*	2.44 ± 0.24*
AH419	nUspA2	CT-CRM _{V72Y}	3.07 ± 0.28*	3.30 ± 0.35*	2.83 ± 0.19*
AH420	nUspA2	CT-CRM _{S68Y,V72Y}	2.80 ± 0.25*	2.86 ± 0.43*	2.52 ± 0.18*

*Significantly higher than the nUspA2/PBS group

Groups of five female BALB/c mice were immunized intranasally at weeks 0, 5 2, and 4 with 10µL containing 5µg nUspA2 adjuvanted with 1µg CT (Sigma) or CT mutants. Endpoint antibody titers were determined from pooled mucosal wash samples collected at week 6. Data are presented as the reciprocal dilution resulting in an OD₄₀₅ of 0.1. The results are shown in Table 25.

Table 27. The mucosal anti-nUspA2 responses of BALB/c mice after intranasal immunization with nUspA2 adjuvanted with mutant CTs

Group	Antigen (5µg)	Adjuvant (1 µg)	Bronch Wash		Nasal Wash		Vaginal Wash		Saliva	
			IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA
AG414	nUspA2	PBS	<10	<10	<10	<10	<10	<10	<10	<10
AG415	nUspA2	CT	<10	<10	<10	34	<10	35	<10	33
AG416	nUspA2	CT-CRM _{E29H}	<10	<10	<10	22	<10	<10	<10	<10
AG417	nUspA2	CT-CRM _{I16A}	<10	<10	<10	<10	<10	17	<10	22
AG418	nUspA2	CT- CRM _{I16A,S68Y}	<10	<10	<10	23	<10	12	<10	17
AG419	nUspA2	CT-CRM _{V72Y}	<10	16	<10	15	<10	58	<10	46
AG420	nUspA2	CT- CRM _{S68Y,V72Y}	<10	<10	<10	14	<10	41	<10	43

5

All publications and references cited in this specification are incorporated herein by reference. While the invention has been described with reference to a particularly preferred embodiment, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

CLAIMS:

1. An immunogenic, mutant cholera holotoxin (CT-CRM) comprising an amino acid sequence of subunit A of the wild-type cholera toxin (CT), wherein said subunit A comprises at least an amino acid substitution in the wild-type CT subunit A amino acid position 16 or position 72, and wherein said mutant CT-CRM has reduced toxicity compared to said wild-type CT.
2. The CT-CRM according to claim 1, comprising a single amino acid substitution wherein said substitution is located at amino acid position 16.
3. The CT-CRM according to claim 2, comprising a single amino acid substitution wherein the amino acid isoleucine in the amino acid position 16 in the A subunit is substituted with an alanine.
4. The CT-CRM according to claim 1, wherein said subunit A differs from said wild-type CT by an amino acid substitution located at amino acid position 16 and an amino acid substitution located at position 68.
5. The CT-CRM according to claim 4, wherein the amino acid isoleucine in the amino acid position 16 in the A subunit is substituted with an alanine and wherein the amino acid serine in the amino acid position 68 in the A subunit is substituted with an alanine.
6. The CT-CRM according to claim 1, comprising a single amino acid substitution wherein said substitution is located at amino acid position 72.
7. The CT-CRM according to claim 6 comprising a single amino acid substitution wherein the amino acid valine in the amino acid position 72 in the A subunit is substituted with a tyrosine.

8. The CT-CRM according to claim 1, wherein said subunit A differs from said wild-type CT by an amino acid substitution located at amino acid position 72 and an amino acid substitution located at position 68.

9. The CT-CRM according to claim 8, wherein the amino acid serine in the amino acid position 68 in the A subunit is substituted with an alanine, and wherein the amino acid valine in the amino acid position 72 in the A subunit is substituted with a tyrosine.

10. The CT-CRM according to claim 1, further comprising at least one additional mutation in the A subunit of the cholera holotoxin at an amino acid position other than the amino acid positions 16, 68 and 72 in the A subunit.

11. The CT-CRM according to claim 10, wherein the one additional mutation is a substitution for a subunit A amino acid selected from the group consisting of the arginine at amino acid position 7, the aspartic acid at amino acid position 9, the arginine at amino acid position 11, the glutamic acid at position 29, the histidine at amino acid position 44, the valine at amino acid position 53, the arginine at amino acid position 54, the serine at amino acid position 61, the serine at amino acid position 63, the histidine at amino acid position 70, the valine at amino acid position 97, the tyrosine at amino acid position 104, the proline at amino acid position 106, the histidine at amino acid position 107, the serine at amino acid position 109, the glutamic acid at amino acid position 110, the glutamic acid at amino acid position 112, the serine at amino acid position 114, the tryptophan at amino acid position 127, the arginine at amino acid position 146, and the arginine at amino acid position 192.

12. An immunogenic composition comprising a mutant cholera holotoxin (CT-CRM) of any of claims 1 through 11, wherein the mutant holotoxin enhances the immune response in a vertebrate host to an antigen.

13. The composition according to claim 12, further comprising an antigen derived from the member of the group consisting of a pathogenic bacterium, virus, fungus or parasite, a cancer cell, a tumor cell, an allergen and a self-molecule.

14. The composition according to claim 13, wherein the selected bacterial antigen is a protein, polypeptide, peptide or fragment derived from a protein.

15. The composition according to claim 13, wherein the bacterial antigen is selected from the bacterial species consisting of typable and non-typable *Haemophilus influenzae*, *Haemophilus somnus*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus faecalis*, *Helicobacter pylori*, *Neisseria meningitidis*, *Neisseria gonorrhoea*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Bordetella pertussis*, *Alloiococcus otiditis*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella choleraesuis*, *Escherichia coli*, *Shigella*, *Vibrio cholerae*, *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, *Mycobacterium avium-Mycabacterium intracellulare complex*, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Clostridium tetani*, *Leptospira interrogans*, *Borrelia burgdorferi*, *Pasteurella haemolytica*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* and *Mycoplasma galliseptum*.

16. The antigenic composition according to claim 15, wherein the *Haemophilus influenzae* antigen is selected from the group consisting of the *Haemophilus influenzae* P4 outer membrane protein, the *Haemophilus influenzae* P6 outer membrane protein and *Haemophilus influenzae* adherence and penetration protein (Hap_s).

17. The composition according to claim 15, wherein the *Helicobacter Pylori* antigen is the *Helicobacter pylori* urease protein.

18. The composition according to claim 15, wherein the *Neisseria meningitidis* antigen is selected from the group consisting of the *Neisseria meningitidis* Group B recombinant class 1 pilin (rpilin) and the *Neisseria meningitidis* Group B class 1 outer membrane protein (PorA).
19. The composition according to claim 13, further comprising an antigen of a pathogenic virus.
20. The composition according to claim 19, wherein the selected viral antigen is a protein, polypeptide, peptide or fragment derived from a protein.
21. The composition according to claim 20, wherein the viral antigen is selected from the viral species consisting of Respiratory syncytial virus, Parainfluenza virus types, 1,2,3, Human metapneumovirus, Influenza virus, Herpes simplex virus, Human cytomegalovirus, Human immunodeficiency virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Human papillomavirus, poliovirus, rotavirus, caliciviruses, measles virus, mumps virus, Rubella virus, adenovirus, rabies virus, canine distemper virus, rinderpest virus, avian pneumovirus (formerly turkey rhinotracheitis virus), Hendra virus, Nipah virus, coronoavirus, parvovirus, infectious rhinotracheitis viruses, feline leukemia virus, feline infectious peritonitis virus, avian infectious bursal disease virus, Newcastle disease virus, Marek's disease virus, porcine respiratory and reproductive syndrome virus, equine arteritis virus and the encephalitis viruses.
22. The composition according to claim 21, wherein the respiratory syncytial virus antigen is the respiratory syncytial virus fusion protein.
23. The composition according to claim 21, wherein the herpes simplex virus (HSV) antigen is the herpes simplex virus (HSV) type 2 glycoprotein D (gD2).
24. The composition according to claim 13, further comprising an antigen from a pathogenic fungus.

25. The composition according to claim 24, wherein the selected fungal antigen is a protein, polypeptide, peptide or fragment derived from a protein.
26. The composition according to claim 24, wherein the fungal antigen is from a fungus selected from the group of pathogenic fungi consisting of *Aspergillus*, *Blastomyces*, *Candida*, *Coccidioides*, *Cryptococcus* and *Histoplasma*.
27. The composition according to claim 13, further comprising an antigen from a pathogenic parasite.
28. The composition according to claim 27, wherein the selected parasite antigen is a protein, polypeptide, peptide or fragment derived from a protein.
29. The composition according to claim 27, wherein the parasite antigen is from a parasite selected from the group of pathogenic parasites consisting of *Leishmania major*, *Ascaris*, *Trichuris*, *Giardia*, *Schistosoma*, *Cryptosporidium*, *Trichomonas*, *Toxoplasma gondii* and *Pneumocystis carinii*.
30. The composition according to claim 13, wherein said antigen is derived from a cancer cell or tumor cell.
31. The composition according to claim 30, wherein said cancer or tumor cell antigen is selected from the group consisting of prostate specific antigen, carcino-embryonic antigen, MUC-1, Her2, CA-125, MAGE-3, a hormone, and a hormone analogs.
32. The composition according to claim 13, wherein said antigen is a polypeptide, peptide or fragment derived from amyloid precursor protein, or an allergen.

33. The composition according to claim 32, wherein the amyloid precursor protein antigen is the A_β peptide, which is a 42 amino acid fragment of amyloid precursor protein, or a fragment of the A_β peptide.
34. The composition according to claim 13, further comprising a diluent, excipient or carrier.
35. The composition according to claim 12, further comprising a second adjuvant in addition to the mutant cholera holotoxin.
36. A method for enhancing the immune response of a vertebrate host to an antigen, said method comprising administering to the host the composition of claim 12 or 13.
37. An isolated and purified DNA sequence encoding an immunogenic, mutant cholera holotoxin of any of claims 1-11.
38. A nucleic acid molecule comprising an isolated and purified nucleic acid sequence encoding an immunogenic, mutant cholera holotoxin of any of claims 1-11, and wherein the sequence encoding the immunogenic, mutant cholera holotoxin is operatively linked to regulatory sequences enabling expression of said mutant holotoxin in a host cell.
39. The molecule according to claim 38, wherein said regulatory sequence is an inducible promoter.
40. The molecule according to claim 38, wherein said promoter is the arabinose inducible promoter.
41. The molecule according to claim 38, wherein said molecule is a viral or non-viral vector.

42. The molecule according to claim 41, wherein said non-viral vector is a DNA plasmid.

43. A host cell transformed, transduced, infected or transfected with the nucleic acid molecule of claim 38.

44. A method of producing an immunogenic mutant cholera holotoxin, wherein the cholera holotoxin has reduced toxicity compared to a wild-type cholera holotoxin and has a single amino acid substitution in the A subunit of the cholera holotoxin, comprising transforming, infecting, transducing or transfecting a host cell with the nucleic acid molecule according to claim 38, and culturing the host cell under conditions which permit the expression of said recombinant immunogenic detoxified protein by the host cell.

45. Use of an effective adjuvanting amount of a mutant cholera holotoxin according to any of claims 1 to 11, in combination with a selected antigen from a pathogenic bacterium, virus, fungus, parasite, a cancer cell, a tumor cell, and allergen, a self molecule, or vertebrate antigen to prepare an immunogenic composition, wherein said mutant holotoxin enhances the immune response in a vertebrate host to said antigen.

SEQUENCE LISTING

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<120> Mutant Forms of Cholera Holotoxin As An Adjuvant

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<150> US 60/296,537
<151> 2001-06-07

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<170> PatentIn version 3.1

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35 40 45

Phe Asp Arg Gly Thr Gln Met Asn Ile Asn Leu Tyr Asp His Ala Arg
50 55 60

Gly Thr Gln Thr Gly Phe Val Arg His Asp Asp Gly Tyr Val Ser Thr
65 70 75 80

Ser Ile Ser Leu Arg Ser Ala His Leu Val Gly Gln Thr Ile Leu Ser
85 90 95

Gly His Ser Thr Tyr Tyr Ile Tyr Val Ile Ala Thr Ala Pro Asn Met
100 105 110

Phe Asn Val Asn Asp Val Leu Gly Ala Tyr Ser Pro His Pro Asp Glu
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Gln Glu Val Ser Ala Leu Gly Gly Ile Pro Tyr Ser Gln Ile Tyr Gly
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Trp Tyr Arg Val His Phe Gly Val Leu Asp Glu Gln Leu His Arg Asn
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Arg Gly Tyr Arg Asp Arg Tyr Tyr Ser Asn Leu Asp Ile Ala Pro Ala
165 170 175

Ala Asp Gly Tyr Gly Leu Ala Gly Phe Pro Pro Glu His Arg Ala Trp
180 185 190

Arg Glu Glu Pro Trp Ile His His Ala Pro Pro Gly Cys Gly Asn Ala
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Pro Arg Ser Ser Met Ser Asn Thr Cys Asp Glu Lys Thr Gln Ser Leu
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Gly Val Lys Phe Leu Asp Glu Tyr Gln Ser Lys Val Lys Arg Gln Ile
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Phe Ser Gly Tyr Gln Ser Asp Ile Asp Thr His Asn Arg Ile Lys Asp
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Glu Leu Met Ile Lys Leu Lys Phe Gly Val Phe Phe Thr Val Leu Leu
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Ser Ser Ala Tyr Ala His Gly Thr Pro Gln Asn Ile Thr Asp Leu Cys
275 280 285

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Ser Tyr Thr Glu Ser Leu Ala Gly Lys Arg Glu Met Ala Ile Ile Thr
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Phe Lys Asn Gly Ala Ile Phe Gln Val Glu Val Pro Ser Ser Gln His
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Ile Asp Ser Gln Lys Lys Ala Ile Glu Arg Met Lys Asp Thr Leu Arg
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Arg Gly Thr Gln Met Asn Ile Asn Leu Tyr Asp His Ala Arg Gly Thr
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Gln Thr Gly Phe Val Arg His Asp Asp Gly Tyr Val Ser Thr Ser Ile
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Ser Leu Arg Ser Ala His Leu Val Gly Gln Thr Ile Leu Ser Gly His
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Ser Thr Tyr Tyr Ile Tyr Val Ile Ala Thr Ala Pro Asn Met Phe Asn
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Val Asn Asp Val Leu Gly Ala Tyr Ser Pro His Pro Asp Glu Gln Glu
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Val Ser Ala Leu Gly Gly Ile Pro Tyr Ser Gln Ile Tyr Gly Trp Tyr
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Arg Val His Phe Gly Val Leu Asp Glu Gln Leu His Arg Asn Arg Gly
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Tyr Arg Asp Arg Tyr Tyr Ser Asn Leu Asp Ile Ala Pro Ala Ala Asp
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Gly Tyr Gly Leu Ala Gly Phe Pro Pro Glu His Arg Ala Trp Arg Glu

165

170

175

Glu Pro Trp Ile His His Ala Pro Pro Gly Cys Gly Asn Ala Pro Arg
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Ser Ser Met Ser Asn Thr Cys Asp Glu Lys Thr Gln Ser Leu Gly Val
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